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14. ABSTRACT Organophosphorus esters (OP) are known to make a covalent bond with the active site serine in the consensus sequence GXSXG of esterases and proteases. However, the site of attachment to proteins that have no active site serine has only recently been recognized as tyrosine. In last year's report we provided mass spectrometry evidence that soman, sarin, DFP, chlorpyrifos oxon, dichlorvos, and FP-biotin bound to tyrosine in 3 proteins. We now report binding of organophosphorus agents to tyrosinein 12 proteins. This suggests that diagnosis of exposure to OP may become possible by monitoring adducts on tyrosine, for example in albumin. The advantages of using organophosphorus-labeled albumin as a biomarker of exposure are that the albumin adduct is stable, and the OPalbumin adduct does not age. The soman adduct on plasma butyrylcholinesterase was used to determine the limit of detection of soman exposure ex vivo. A concentration of soman that inhibited butyrylcholinesterase in human plasma 2% was detectable in the multiple reaction monitoring mode of the mass spectrometer.

15. SUBJECT TERMS
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Ding SJ, Carr J, Carlson JE, Tong L, Xue W, Li Y, Schopfer LM, Li B, Nachon F, Asojo O, Thompson CM, Hinrichs SH, Masson P, Lockridge O. Five tyrosines and two serines in human albumin are labeled by the organophosphorus agent FP-biotin. Chem Res Toxicol. 2008 Sep;21(9):1787-94. Epub 2008 Aug 16. PMID: 18707141

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Grigoryan H, Li B, Anderson EK, Xue W, Nachon F, Lockridge O, Schopfer LM. Covalent binding of the organophosphorus agent FP-biotin to tyrosine in eight proteins that have no active site serine. Chem Biol Interact 2009 in press

ABBREVIATIONS

AChE	acetylcholinesterase
amu	atomic mass unit
BChE	butyrylcholinesterase
CAM	carbamidomethylated cysteine
CHCA	alpha-cyano 4-hydoxycinnamic acid; matrix for MALDI
CID	collision induced dissociation
CPO	chlorpyrifos oxon; O,O-diethyl-(3,5,6-trichloro-2-pyridyl) phosphate
DCV	dichlorvos, O,O-dimethyl-(2,2-dichloroethenyl) phosphate
DFP	diisopropylfluorophosphate
FENTA	ferric nitrilotriacetate
FPB	FP-biotin
FP-biotin	biotin-tagged organophosphorus agent; 10-fluoroethoxyphosphinyl-N-biotinamidopentyldecanamide
HPLC	high performance liquid chromatography
LC/MS/MS	liquid chromatography coupled to tandem mass spectrometry
MALDI-TOF	matrix assisted laser desorption ionization-time of flight mass spectrometer
MRM	multiple reaction monitoring
MS/MS	tandem mass spectrometry in which the mass of a molecule is determine in the first MS and the masses of the fragments are determined in the second MS
m/z	mass to charge ratio
OP	organophosphorus toxicant
PAGE	polyacrylamide gel electrophoresis
PVDF	polyvinylidene fluoride membrane for protein blots
sarin	O-isopropylmethylphosphonofluoridate
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
soman	O-pinacolylmethylphosphonofluoridate
TFA	trifluoroacetic acid
UCSF	University of California San Francisco
Y	single letter code for tyrosine

INTRODUCTION

There is overwhelming evidence that the acute toxicity of organophosphorus nerve agents and pesticides (OP) is due to inhibition of acetylcholinesterase. The OP binds covalently to the active site serine to make an irreversible adduct. The OP-modified acetylcholinesterase is incapable of performing its physiological function of hydrolyzing the neurotransmitter acetylcholine. Excess acetylcholine overstimulates receptors, initiating a cascade of reactions that results in uncontrolled seizures and respiratory arrest (McDonough and Shih, 1997).

The active site serine of acetylcholinesterase is located in a conserved sequence GlyXSerXGly common to all serine esterases and serine proteases.

What is not understood is why some people suffer chronic illness from a dose of OP too low to inhibit acetylcholinesterase. The implication is that OP reactive proteins exist that are more sensitive than acetylcholinesterase to OP. When we began this work we assumed that these highly reactive proteins would have serine at the active site. But when we searched for these proteins using a biotinylated OP we consistently found proteins that had no active site serine. At first we suspected that the avidin beads had nonspecifically bound these proteins and that we were looking at artifacts. To convince ourselves that a protein like tubulin actually binds OP, we studied OP binding to pure tubulin. We used mass spectrometry to identify the OP binding site. The labeled amino acid was tyrosine. We studied other pure proteins, and finally synthetic peptides. A consistent pattern emerged of OP binding to tyrosine.

We believe that we have identified a new motif for OP binding to proteins. This report presents the evidence for our conclusion that OP bind not only to serine hydrolases but also to proteins that contain an activated tyrosine.

A potential use of this new information is in diagnosis of exposure to organophosphorus agents. Nerve agent adducts on the serine of acetylcholinesterase and butyrylcholinesterase lose the alkyl group that distinguishes soman, sarin, cyclosarin, and VX. In contrast, nerve agent adducts on tyrosine of albumin are stable and do not age. This will make it possible to positively identify the neurotoxic agent.

APPROVED STATEMENT OF WORK

Statement of work

Year 1

Task 1. Determine the characteristic fragmentation pattern of soman covalently bound to a serine hydrolase (trypsin). *Completed in years 1 and 2*

Task 2. Determine the characteristic fragmentation pattern of soman covalently bound to a tyrosine esterase (albumin). *Completed in years 1 and 2. Three published papers.*

Li B, Nachon F, Froment MT, Verdier L, Debouzy JC, Brasme B, Gillon E, Schopfer LM, Lockridge O, Masson P. Binding and hydrolysis of soman by human serum albumin. Chem Res Toxicol. 2008 Feb;21(2):421-31. Epub 2007 Dec 29. PMID: 18163544

Grigoryan H, Schopfer LM, Thompson CM, Terry AV, Masson P, Lockridge O. Mass spectrometry identifies covalent binding of soman, sarin, chlorpyrifos oxon, diisopropyl fluorophosphate, and FP-biotin to tyrosines on tubulin: a potential mechanism of long term toxicity by organophosphorus agents. Chem Biol Interact. 2008 Sep 25;175(1-3):180-6. Epub 2008 Apr 22. PMID: 18502412

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Task 3. Identify the proteins in human plasma that bind the nerve agent simulant, FP-biotin. *Progress in years 1 and 2. Two published papers; one in press*

Ding SJ, Carr J, Carlson JE, Tong L, Xue W, Li Y, Schopfer LM, Li B, Nachon F, Asojo O, Thompson CM, Hinrichs SH, Masson P, Lockridge O. Five tyrosines and two serines in human albumin are labeled by the organophosphorus agent FP-biotin. Chem Res Toxicol. 2008 Sep;21(9):1787-94. Epub 2008 Aug 16. PMID: 18707141

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Grigoryan H, Li B, Anderson EK, Xue W, Nachon F, Lockridge O, Schopfer LM. Covalent binding of the organophosphorus agent FP-biotin to tyrosine in eight proteins that have no active site serine. Chem Biol Interact 2009 in press

Year 2

Task 4. Set up a Multiple Reaction Monitoring method to identify soman-labeled proteins, using purified proteins. *Progress in year 2. One published paper.*

Li H, Ricordel I, Tong L, Schopfer LM, Baud F, Mégarbane B, Maury E, Masson P, Lockridge O. Carbofuran poisoning detected by mass spectrometry of butyrylcholinesterase adduct in human serum. J Appl Toxicol. 2009 Mar;29(2):149-55. PMID: 18937214

Task 5. Use Multiple Reaction Monitoring to identify soman-labeled proteins in human plasma.

Task 6. Use a second method, for example enzyme activity assays or immunoprecipitation, to confirm the identity of soman-labeled proteins from plasma. *Progress in year 2. One published paper.*

Li B, Schopfer LM, Grigoryan H, Thompson CM, Hinrichs SH, Masson P, Lockridge O. Tyrosines of human and mouse transferrin covalently labeled by organophosphorus agents: a new motif for binding to proteins that have no active site serine. Toxicol Sci. 2009 Jan;107(1):144-55. Epub 2008 Oct 16. PMID: 18930948. *This publication fulfills Tasks 3 and 6.*

Task 7. Determine the limit of detection of soman-labeled proteins in human plasma. *Progress in year 2.*

Task 1. Determine the characteristic fragmentation pattern of soman covalently bound to a serine hydrolase (trypsin).

Relation to statement of work. Task 1 has been completed.

The fragmentation patterns of tryptic peptides of soman-labeled bovine trypsin and human butyrylcholinesterase

ABSTRACT

The goal was to search for characteristic fragment ions that would indicate covalent binding of soman to serine in proteins. Two highly purified proteins, bovine trypsin and human butyrylcholinesterase, were labeled with soman and their tryptic peptides analyzed by mass spectrometry. Aged soman adducts on serine that had lost the pinacolyl group were found for both proteins. MSMS spectra of the butyrylcholinesterase adduct showed dehydroalanine ions, where the active site serine had been converted to dehydroalanine. In contrast, dehydroalanine ions were not found in soman-labeled trypsin. To aid in identification of soman-labeled peptides, we introduced O-pinacolylmethylphosphonate as a variable modification option in the Mascot search engine. This variable modification option is available to all users of Mascot.

INTRODUCTION

The nerve agent soman is lethal because it disrupts nerve impulse transmission. Soman toxicity is initiated by irreversible inhibition of acetylcholinesterase in cholinergic nerve synapses. Butyrylcholinesterase and carboxylesterase are also inhibited by soman, but inhibition of these esterases has no apparent health consequences. Trypsin is inhibited by soman, but only in ex vivo experiments at high concentrations of soman. The reaction of soman with the active site serine of acetylcholinesterase, butyrylcholinesterase, and trypsin is illustrated in Figure 1.1. Soman makes a covalent bond with the active site serine in a process that releases the fluoride ion from soman. The soman-serine adduct undergoes a dealkylation reaction called aging, in which the pinacolyl group of soman is released. Aging is an enzyme catalyzed reaction in acetylcholinesterase and in trypsin (Viragh et al., 1997). Carboxylesterase does not undergo aging (Maxwell and Brecht, 2001).

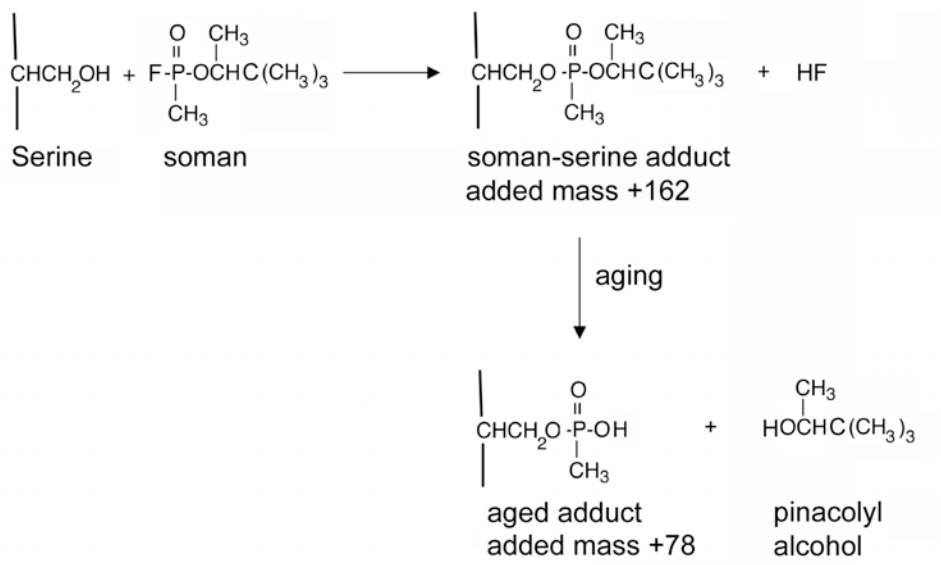


Figure 1.1. Covalent binding of soman to the active site serine of acetylcholinesterase, butyrylcholinesterase, and trypsin. The initial step releases fluoride from soman and adds a mass of +162 amu to the protein. This is followed by an aging step that releases pinacolyl alcohol from soman. The aged soman adduct has an added mass of +78 amu.

It is expected that many proteins in addition to acetylcholinesterase, butyrylcholinesterase, and carboxylesterase bind soman covalently on serine in live animals treated with sublethal doses of soman (Traub, 1985; Little et al., 1988). To be able to identify these proteins one needs a marker. Radiolabeled soman would be an excellent marker, but radiolabeled soman is not available. A reasonable alternative when analyzing peptides by mass spectrometry is the presence of a characteristic fragment ion in the MSMS spectrum. The goal of the present work was to search for characteristic fragment ions that would indicate covalent binding of soman to serine. The results of this study are useful when searching for unknown proteins that covalently bind soman to serine.

We used highly purified bovine trypsin and human butyrylcholinesterase as model serine hydrolases because these proteins are known to bind soman on the active site serine (Bencsura et al., 1995; Millard et al., 1999; Fidder et al., 2002; Tsuge and Seto, 2006). Soman-labeled butyrylcholinesterase is particularly interesting because butyrylcholinesterase is abundant in human plasma and is therefore useful as a biomarker of exposure.

METHODS

Materials. Human butyrylcholinesterase was purified from outdated human plasma as described (Lockridge et al., 2005). The 72% pure preparation was in 0.15M NaCl, 10 mM potassium phosphate pH 7.4, and had an activity of 830 units/ml, which calculates to a butyrylcholinesterase concentration of 1.15 mg/ml (13.5 μM). The subunit molecular weight of butyrylcholinesterase is 85,000. Bovine trypsin was from Sigma (St. Louis, Missouri) catalog #T-8642, TPCK treated, from bovine pancreas. A stock solution of 2.2 mg/ml was prepared in

50 mM acetic acid. The molecular weight of trypsin is 23,474. Racemic soman (pinacolyl methylfluorophosphonate) from CEB (Vert-le-Petite, France) was diluted in isopropanol. Labeling of butyrylcholinesterase and albumin with authentic soman was performed in a certified military laboratory. The soman analog, 3-chloro-4-methyl-2-oxo-2H-chromen-7-yl 1,1,1-trimethylpropyl methylphosphonate, was a gift from Gareth Williams (Briseno-Roa et al., 2006). It is compound #11 in Table 2 of the paper by (Briseno-Roa et al., 2006). A 0.1 M solution was prepared in anhydrous acetonitrile and used immediately to label butyrylcholinesterase and trypsin. The soman analog makes exactly the same adducts as authentic soman. However, it is less toxic and can therefore be used in nonmilitary laboratories

Butyrylcholinesterase labeled with authentic soman. A 0.5 ml aliquot of 1.15 mg/ml butyrylcholinesterase (13.5 μM) in phosphate buffered saline pH 7.4 was treated with 200 μM soman. The sample was stored for two weeks at room temperature to allow excess soman to completely decay.

Tryptic peptides of soman-labeled butyrylcholinesterase. The soman-treated butyrylcholinesterase was desalted by dialysis in a Slide-A-Lyzer 7K dialysis cassette (Pierce, Rockford, IL; catalog # 66370) against 2 x 4 L of 10 mM ammonium bicarbonate pH 8.3. The protein was desalted to make it possible to estimate digestion efficiency in the MALDI-TOF mass spectrometer before proceeding to additional peptide purification steps. Essentially salt-free samples are required for MALDI-TOF mass spectrometry.

Butyrylcholinesterase was denatured by incubation for 10 min in a boiling water bath. The 574 μg of denatured butyrylcholinesterase in 0.5 ml of 10 mM ammonium bicarbonate pH 8.3 was digested with 20 μg of sequencing grade modified porcine trypsin (Promega, Madison, WI; catalog # V5113) overnight at 37˚C. Digestion efficiency was checked by MALDI-TOF mass spectrometry.

Standard methods of denaturation in 8 M urea followed by reduction of disulfide bonds, alkylation of sulfhydryl groups with iodoacetamide, dialysis, and trypsin digestion were performed on another sample. These additional steps did not increase the yield of labeled active site peptide and were therefore omitted.

Tryptic peptides of soman-labeled butyrylcholinesterase were separated by offline HPLC on a C18 reverse phase column (Phenomenex Prodigy 5 micron ODS(2) size 100 x 4.60 mm part No. OOD-3300-EO S/N 216078-6) using a 60 min gradient (buffer A = 0.1% trifluoroacetic acid; buffer B =100% acetonitrile, 0.08% trifluoroacetic acid) that started with 100% buffer A at time zero and increased to 60% buffer B at 60 min. The flow rate was 1 ml/min. Absorbance at 210 nm was recorded. The HPLC system was Waters 625.

Trypsin and butyrylcholinesterase labeled with soman analog. A 13 μl aliquot of 2.2 mg/ml bovine trypsin in 50 mM acetic acid (1.2 nanomoles) was diluted with 175 μl of 50 mM ammonium bicarbonate to bring the pH to 8.2. Labeling was initiated by addition of 12 μl of 0.1 M soman analog in acetonitrile (1200 nanomoles). The trypsin solution became turbid when the soman analog was added, but clarified after overnight incubation at 37˚C.

A 0.05 mg aliquot of butyrylcholinesterase (0.58 nanomoles) was treated with 100 nanomoles of soman analog. Butyrylcholinesterase activity was completely inhibited within the first time point tested, 10 min after addition of soman analog.

Tryptic peptides of soman-labeled trypsin. Since the soman analog produces the exact same soman-labeled derivative as authentic soman, the labeled trypsin is referred to as soman-labeled trypsin. The soman-labeled trypsin was diluted to 0.5 ml by adding 0.3 ml of water to 0.2 ml reaction mixture. The protein was denatured by adding 0.4 g solid urea to make the solution 8 M urea. Disulfide bonds were reduced by treating with 0.1 ml of 0.1 M dithiothreitol for 10 min in a boiling water bath. After the solution had cooled, the sulfhydryl groups were alkylated with 0.02 g of solid iodoacetamide (MW 185.0) to make the solution 0.1 M iodoacetamide. After 1 h incubation in the dark the sample was desalted by dialysis against 3 x 4 L of 10 mM ammonium bicarbonate pH 8.3. The dialyzed soman-trypsin was reduced in volume to about 50 µl in a vacuum centrifuge. The 28 µg of soman-trypsin was digested with 1 µl of 0.4 µg/µl trypsin (Promega, catalog # V5113) overnight at 37˚C.

MALDI-TOF and MALDI-TOF-TOF mass spectrometry. MS and MSMS spectra were acquired on the MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems, Foster City, CA). Essentially salt-free 0.5 µl samples were spotted on a MALDI target plate (Opti-TOF 384 well insert, part number 1016491, Applied Biosystems). The samples were air dried and overlaid with 0.5 µl of 10 mg/ml alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid. Spectra were acquired with laser power at 4000 V in positive reflector mode. The mass spectrometer was calibrated against des-Arg-Bradykinin, Angiotensin 1, Glu fibrinopeptide B and neurotensin (Cal Mix 1, Applied Biosystems). Each spectrum was the average of 500 laser shots.

Infusion in the QTRAP 4000 mass spectrometer. HPLC purified OP-labeled tryptic peptides were analyzed by this method when the mass of the parent ion was known from MALDI-TOF experiments. Peptides were dissolved in 50% acetonitrile, 0.1% formic acid to a concentration of 2-6 pmol/µl. Peptides were infused into the QTRAP 4000 (Applied Biosystems) mass spectrometer at a flow rate of 0.3 µl/min through an 8 µm emitter (#FS360-50-8-D, New Objective) via a 25 µl Hamilton syringe mounted on a Harvard pump. Five hundred MSMS spectra were accumulated for each parent ion.

RESULTS

Soman-labeled butyrylcholinesterase. The expected masses of the tryptic peptide of butyrylcholinesterase labeled with soman are listed in Table 1.1. The peptide without a label has a mass of 2928.5 amu. After modification by soman the mass is 3090.5 amu, and after aging the mass is 3006.5 amu.

Table 1.1. Expected masses of soman-labeled peptides of human butyrylcholinesterase

name	accession#	sequence	mass no label	mass +162	mass +78
butyryl cholinesterase	gi:116353	SVTLFGE**S**AGAASVSLHLLSPGSHSLFTR	2928.5	3090.5	3006.5

The active site serine is indicated by the large S. Soman adds a mass of 162. Aged soman adds a mass of 78 to the active site serine.

MALDI-TOF analysis of HPLC fractions showed that a peptide with a mass of 3006.5 eluted with 34-35% acetonitrile. No peptide with a mass of 3090.5 was detected. The HPLC fraction containing the 3006.5 amu peptide was infused into the QTRAP mass spectrometer where it was fragmented by collision induced dissociation. Figure 1.2 shows the MSMS spectrum for the triply charged parent ion of 1004.0 m/z.

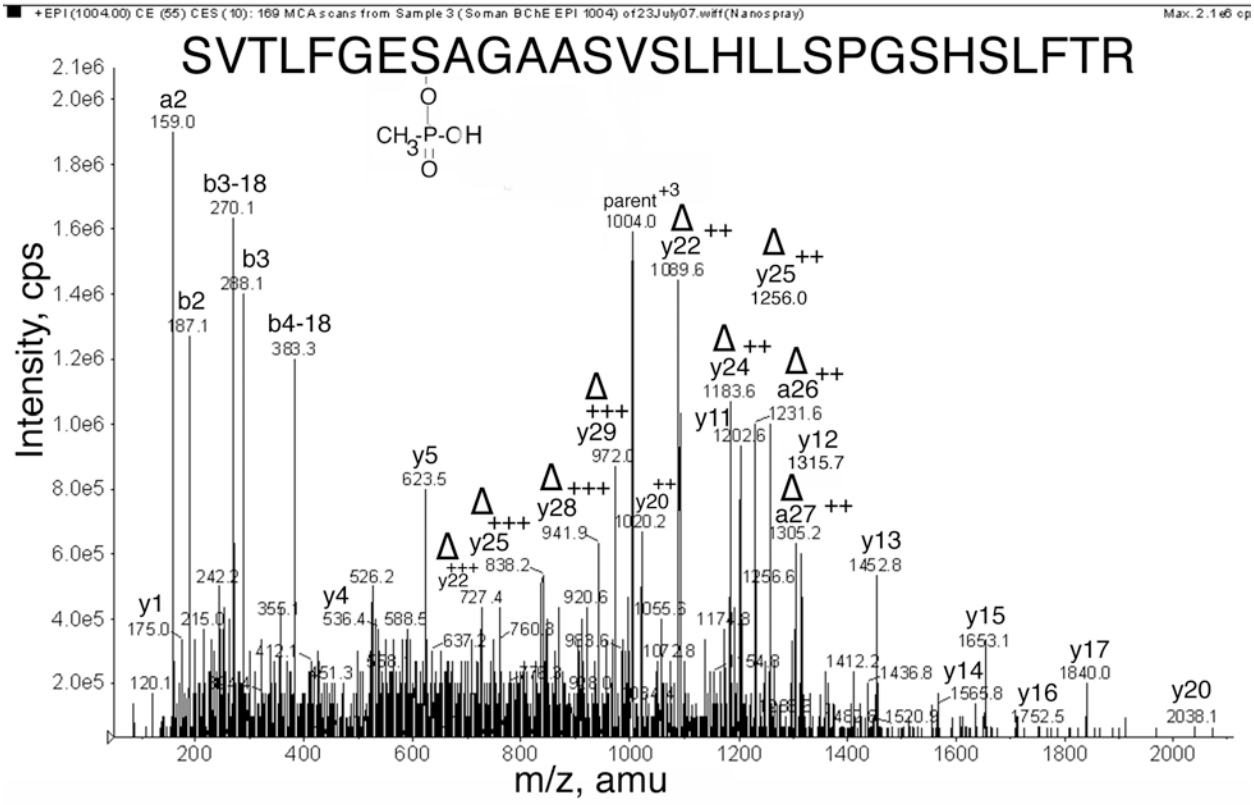


Figure 1.2. MSMS spectrum of aged soman-labeled butyrylcholinesterase tryptic peptide. The parent ion at 1004.0 m/z is triply charged. Dehydroalanine forms of the peptide are indicated by the symbol Δ. The MSMS spectrum was acquired on the QTRAP 4000 mass spectrometer.

The ions in Figure 1.2 support the sequence SVTLFGESAGAASVSLHLLSPGSHSLFTR. The mass of the parent ion is consistent with the presence of aged soman in the peptide. Support for the location of aged soman on serine 8 in the peptide is provided by the nine dehydroalanine ions indicated by the symbol Δ. Dehydroalanine is formed from serine by loss of the OP plus loss of a molecule of water. Figure 1.3 illustrates the mechanism of conversion of organophosphonylated serine to dehydroalanine in a beta-elimination reaction.

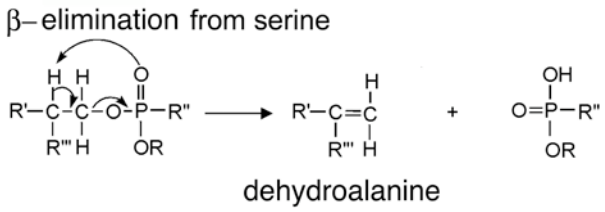


Figure 1.3. Beta-elimination converts OP-labeled serine to dehydroalanine.

Dehydroalanine ions include the triply charged y22+++ to y29+++ ions. These ions indicate that the serine is modified in the parent ion, but has been converted to dehydroalanine by the energy of fragmentation in the mass spectrometer. No ions in Figure 1.2 carry aged soman, though other spectra for the doubly charged parent ion do show small y22 -y25 ions with an added mass of +78 amu.

In summary, the characteristic feature of the MSMS spectrum of soman-labeled butyrylcholinesterase is the presence of dehydroalanine containing ions. A characteristic feature of the parent ion is the presence of the aged soman adduct with an added mass of +78.

Soman-labeled trypsin. Mass spectrometry identified three different trypsin sequences in the bovine trypsin purchased from Sigma. The sequences of the active site tryptic peptides are given in Table 1.2. Peptide DSC*QGDSGGPVVC*SGK with a mass of 1609.7 and peptide GDSC*QGDSGGPVVC*SGK with a mass of 1666.7 carried no soman label. Peptide DSC*QGDSGGPVAC*NGQLQGIVSWGYGC*AQK with a mass of 3234.4 was labeled on the active site serine with aged soman. No unlabeled peptide with mass 3156.4 was found. None of the bovine trypsin peptides carried an added mass of +162 from soman.

Table 1.2. Expected masses of soman-labeled peptides of bovine trypsin

name	accession#	sequence	mass no label	mass +162	mass +78
bovine trypsin	gi:830	DSC*QGDSGGPVAC*NGQLQGIVSWGYGC*AQK	3156.4	3318.4	**3234.4**
bovine trypsin	gi:157878189	GDSC*QGDSGGPVVC*SGK	**1666.7**	1828.7	1744.7
bovine trypsin	gi:165973958	DSC*QGDSGGPVVC*SGK	**1609.7**	1771.7	1687.7

*asterisk indicates carbamidomethylated cysteine. Carbamidomethylation by iodoacetamide adds +57 to each cysteine. The active site serine is indicated by the large S. Soman adds a mass of 162. Aged soman adds a mass of 78 to the active site serine. The masses in bold letters are peptides that we actually found by mass spectrometry.

The most common form of bovine trypsin is represented by accession number gi:165973958. PubMed shows that 213 crystal structures of bovine trypsin with this sequence are deposited in the Protein Data Bank (pdb code 5ptp). The sequence represented by accession number gi:157878189 has also been crystallized (pdb code 1hj9). However, the sequence represented by accession number gi:830 has not been crystallized. This seems to be the rarest form of trypsin. It is odd that this is the only bovine trypsin peptide that was labeled with soman. It suggests that the fluorescent soman analog used for labeling may have selectively bound to this rare form of bovine trypsin. The structure of the fluorescent soman analog is in Figure 1.4. Alternatively, the other soman-labeled tryptic peptides may have spontaneously reactivated.

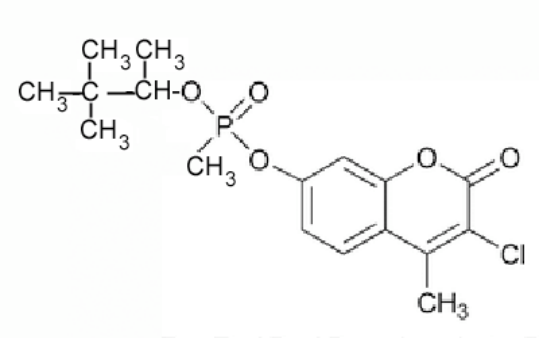


Figure 1.4. Structure of the fluorescent soman analog that was used to label bovine trypsin.

The MSMS spectrum of soman-labeled bovine trypsin is shown in Figure 1.5. The y-ion series from y5 to y25 is consistent with the sequence for the 30 amino acid residue peptide shown in Figure 1.5. The y25 ion is of particular note because the 2687.1 mass includes the methylphosphonate from aged soman. The methylphosphonate has remained bound to serine during the fragmentation procedure. No masses were found for a dehydroalanine form of this peptide.

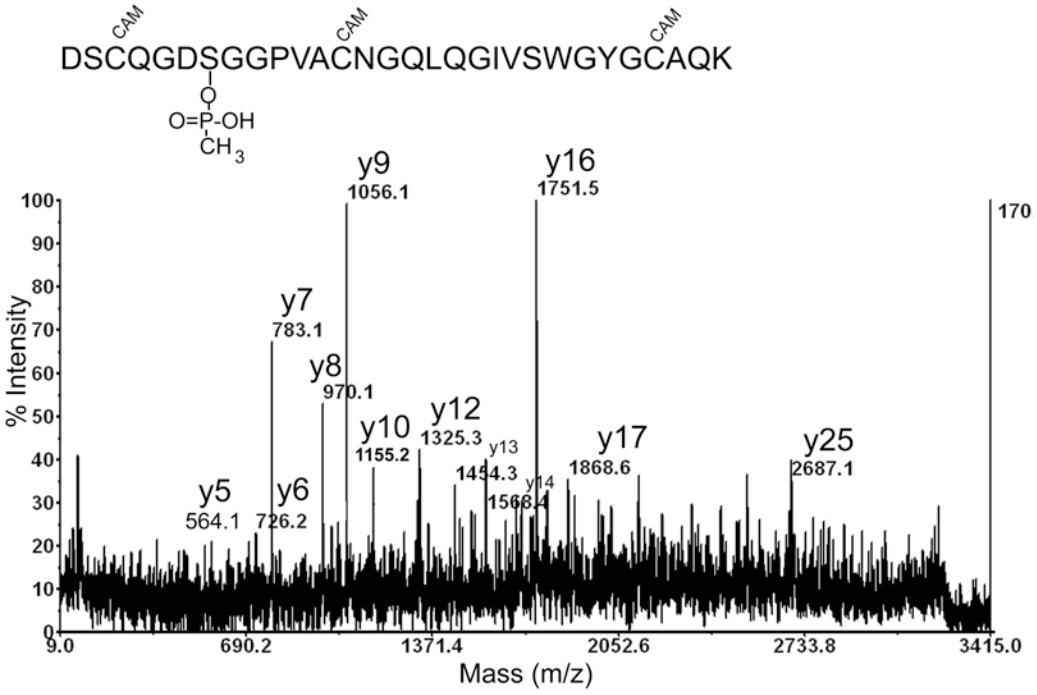


Figure 1.5. Tryptic peptide of bovine trypsin covalently modified on serine with aged soman. The MSMS spectrum of parent ion 3234 was obtained on the MALDI-TOF-TOF mass spectrometer. CAM indicates carbamidomethylated cysteine. The y25 ion includes the mass of aged soman (+78). The accession number for the sequence is gi:830 in the NCBI database.

In summary, the MSMS spectrum of soman-labeled trypsin acquired on the MALDI-TOF-TOF mass spectrometer showed no dehydroalanine ions. The absence of dehydroalanine ions in soman-labeled trypsin is a major difference between the fragmentation of soman-trypsin and soman-butyrylcholinesterase. We have not ruled out the possibility that the difference is due to the use of different types of mass spectrometers. The parent ion of soman-trypsin was similar to the parent ion of soman-butyrylcholinesterase in that the parent ion was the aged soman adduct.

DISCUSSION

Soman-labeled butyrylcholinesterase. Soman-inactivated human butyrylcholinesterase ages with a half-life of about 4 min, similar to the half-life of aging of human acetylcholinesterase (Shafferman et al., 1996; Masson et al., 1997; Saxena et al., 1998). The rapid aging reaction explains why we found only the aged soman adduct of butyrylcholinesterase and none of the nonaged soman adduct.

Residues in the active site gorge of butyrylcholinesterase and acetylcholinesterase assist in the dealkylation of organophosphorus adducts on serine (Masson et al., 1997; Viragh et al., 1997). These residues are absent in trypsin and explain why dealkylation is slow in trypsin.

Soman-labeled trypsin. Soman-inactivated trypsin ages 4 orders of magnitude more slowly than soman-inactivated acetylcholinesterase (Viragh et al., 1997). Soman-inactivated trypsin reactivates faster than it ages. We found strong signals for 2 unlabeled active site peptides of trypsin, and a weak signal for aged soman-labeled trypsin. The strong signals for unlabeled active site peptides are consistent with a fast reactivation rate for soman-labeled trypsin.

How to identify soman-labeled proteins. Comparison of the MSMS spectra of soman-labeled butyrylcholinesterase and trypsin tryptic peptides shows a major difference between them. The butyrylcholinesterase peptide undergoes beta-elimination to convert OP-serine to dehydroalanine. The dehydroalanine ions are highly characteristic of OP-labeling on serine (Fidder et al., 2002). It was expected that OP-trypsin would also have dehydroalanine ions, but this was not found. Similarly, mass spectrometry analysis of DFP-treated chymotrypsin yielded no dehydroalanine ions in the MSMS spectrum (Tsuge and Seto, 2002).

The best way to identify soman-labeled proteins is to do a MASCOT search of MSMS data. To make this possible we have added soman as a variable modification option. The MASCOT modification file is an open source software called UNIMOD. The soman modification on serine, threonine, and tyrosine was introduced according to the instructions found on the web site http://www.unimod.org. Access to the modification is freely available to all MASCOT users in the Variable Modifications menu under the name O-pinacolylmethylphosphonate.

MASCOT does not recognize ions that contain dehydroalanine in place of serine. Dehydroalanine ions often give very intense signals. Assignment of dehydroalanine ions requires manual inspection of MSMS spectra.

Task 2. Determine the characteristic fragmentation pattern of soman covalently bound to a tyrosine esterase (albumin).

Relation to Statement of Work. The manuscript below was reported in the Annual Report for the previous year as a completed Milestone rather than a Task. To make it easier to keep track of completed Tasks, the published paper is included this year under the heading Task 2.

Binding and hydrolysis of soman by human serum albumin

Li B, Nachon F, Froment MT, Verdier L, Debouzy JC, Brasme B, Gillon E, Schopfer LM, Lockridge O, Masson P. Binding and hydrolysis of soman by human serum albumin. Chem Res Toxicol. 2008 Feb;21(2):421-31. Epub 2007 Dec 29. PMID: 18163544

ABSTRACT

Human plasma and fatty acid free human albumin were incubated with soman at pH 8.0 and 25 degrees C. Four methods were used to monitor the reaction of albumin with soman: progressive inhibition of the aryl acylamidase activity of albumin, the release of fluoride ion from soman, 31P NMR, and mass spectrometry. Inhibition (phosphonylation) was slow with a bimolecular rate constant of 15±3 M^{-1} min^{-1}. MALDI-TOF and tandem mass spectrometry of the soman-albumin adduct showed that albumin was phosphonylated on tyrosine 411. No secondary dealkylation of the adduct (aging) occurred. Covalent docking simulations and 31P NMR experiments showed that albumin has no enantiomeric preference for the four stereoisomers of soman. Spontaneous reactivation at pH 8.0 and 25 degrees C, measured as regaining of aryl acylamidase activity and decrease of covalent adduct (pinacolyl methylphosphonylated albumin) by NMR, occurred at a rate of 0.0044 h^{-1}, indicating that the adduct is quite stable ($t_{1/2}$ = 6.5 days). At pH 7.4 and 22 degrees C, the covalent soman-albumin adduct, measured by MALDI-TOF mass spectrometry, was more stable ($t_{1/2}$ = 20 days). Though the concentration of albumin in plasma is very high (about 0.6 mM), its reactivity with soman (phosphonylation and phosphotriesterase activity) is too slow to play a major role in detoxification of the highly toxic organophosphorus compound soman. Increasing the bimolecular rate constant of albumin for organophosphates is a protein engineering challenge that could lead to a new class of bioscavengers to be used against poisoning by nerve agents. Soman-albumin adducts detected by mass spectrometry could be useful for the diagnosis of soman exposure.

A PDF file of the published paper is attached.

Additional progress on Task 2. The following manuscript was included in the Annual Report for the previous year. It has been modified and is now published.

Mass spectrometry identifies covalent binding of soman, sarin, chlorpyrifos oxon, diisopropyl fluorophosphate, and FP-biotin to tyrosines on tubulin: a potential mechanism of long term toxicity by organophosphorus agents

Grigoryan H, Schopfer LM, Thompson CM, Terry AV, Masson P, Lockridge O. Mass spectrometry identifies covalent binding of soman, sarin, chlorpyrifos oxon, diisopropyl fluorophosphate, and FP-biotin to tyrosines on tubulin: a potential mechanism of long term toxicity by organophosphorus agents. Chem Biol Interact. 2008 Sep 25;175(1-3):180-6. Epub 2008 Apr 22. PMID: 18502412

ABSTRACT

Chronic low dose exposure to organophosphorus poisons (OP) results in cognitive impairment. Studies in rats have shown that OP interfere with microtubule polymerization. Since microtubules are required for transport of nutrients from the nerve cell body to the nerve synapse, it has been suggested that disruption of microtubule function could explain the learning and memory deficits associated with OP exposure. Tubulin is a major constituent of microtubules. We tested the hypothesis that OP bind to tubulin by treating purified bovine tubulin with sarin, soman, chlorpyrifos oxon, diisopropylfluorophosphate, and 10-fluoroethoxyphosphinyl-N-biotinamidopentyldecanamide (FP-biotin). Tryptic peptides were isolated and analyzed by mass spectrometry. It was found that OP bound to tyrosine 83 of alpha tubulin in peptide TGTYR, tyrosine 59 in beta tubulin peptide YVPR, tyrosine 281 in beta tubulin peptide GSQQYR, and tyrosine 159 in beta tubulin peptide EEYPDR. The OP reactive tyrosines are located either near the GTP binding site or within loops that interact laterally with protofilaments. It is concluded that OP bind covalently to tubulin, and that this binding could explain cognitive impairment associated with OP exposure.

A PDF file of the published paper is attached.

Additional progress on Task 2. The following manuscript was included in the Annual Report for the previous year. It has been modified and is now published.

Pseudo-esterase activity of human albumin: slow turnover on tyrosine 411 and stable acetylation of 82 residues including 59 lysines

Lockridge O, Xue W, Gaydess A, Grigoryan H, Ding SJ, Schopfer LM, Hinrichs SH, Masson P. Pseudo-esterase activity of human albumin: slow turnover on tyrosine 411 and stable acetylation of 82 residues including 59 lysines. J Biol Chem. 2008 Aug 15;283(33):22582-90. Epub 2008 Jun 24.PMID: 18577514

ABSTRACT

Human albumin is thought to hydrolyze esters because multiple equivalents of product are formed for each equivalent of albumin. Esterase activity with p-nitrophenyl acetate has been attributed to turnover at tyrosine 411. However, p-nitrophenyl acetate creates multiple, stable, acetylated adducts, a property contrary to turnover. Our goal was to identify residues that become acetylated by p-nitrophenyl acetate and determine the relationship between stable adduct formation and turnover. Fatty acid free human albumin was treated with 0.5 mM p-nitrophenyl acetate for 5 min to two weeks, or with 10 mM p-nitrophenyl acetate for 48 h to two weeks. Aliquots were digested with pepsin, trypsin, or GluC and analyzed by mass spectrometry to identify labeled residues. Only Tyr411 was acetylated within the first 5 min of reaction with 0.5 mM p-nitrophenyl acetate. After 0.5-6 h there was partial acetylation of 16-17 residues including Asp1, Lys4, Lys12, Tyr411, Lys413, and Lys414. Treatment with 10 mM p-nitrophenyl acetate resulted in acetylation of 59 lysines, 10 serines, 8 threonines, 4 tyrosines, and Asp1. When Tyr411 was blocked with diisopropylfluorophosphate or chlorpyrifos oxon, albumin had normal esterase activity with beta-naphthyl acetate as visualized on a nondenaturing gel. However, after 82 residues had been acetylated, esterase activity was almost completely inhibited. The half-life for deacetylation of Tyr411 at pH 8.0, 22˚C was 61±4 h. Acetylated lysines formed adducts that were even more stable. In conclusion, the pseudo-esterase activity of albumin is the result of irreversible acetylation of 82 residues and is not the result of turnover.

A PDF file of the published paper is attached.

Task 3. Identify the proteins in human plasma that bind the nerve agent simulant, FP-biotin.

Relation to Statement of Work. The Annual Report for the previous year included a draft of the following manuscript. The manuscript was modified according to reviewers' comments and is now published.

Five tyrosines and two serines in human albumin are labeled by the organophosphorus agent FP-biotin

Ding SJ, Carr J, Carlson JE, Tong L, Xue W, Li Y, Schopfer LM, Li B, Nachon F, Asojo O, Thompson CM, Hinrichs SH, Masson P, Lockridge O. Five tyrosines and two serines in human albumin are labeled by the organophosphorus agent FP-biotin. Chem Res Toxicol. 2008 Sep;21(9):1787-94. Epub 2008 Aug 16. PMID: 18707141

ABSTRACT

Tyrosine 411 of human albumin is an established site for covalent attachment of 10-fluoroethoxyphosphinyl-N-biotinamidopentyldecanamide (FP-biotin), diisopropylfluorophosphate, chlorpyrifos oxon, soman, sarin, and dichlorvos. This work investigated the hypothesis that other residues in albumin could be modified by organophosphorus agents (OP). Human plasma was aggressively treated with FP-biotin; plasma proteins were separated into high and low abundant portions using a proteome partitioning antibody kit, and the proteins were digested with trypsin. The FP-biotinylated tryptic peptides were isolated by binding to monomeric avidin beads. The major sites of covalent attachment identified by mass spectrometry were Y138, Y148, Y401, Y411, Y452, S232 and S287 of human albumin. Prolonged treatment of pure human albumin with chlorpyrifos oxon labeled Y138, Y150, Y161, Y401, Y411 and Y452. To identify the most reactive residue, albumin was treated for 2 h with DFP, FP-biotin, chlorpyrifos oxon, or soman, digested with trypsin or pepsin and analyzed by mass spectrometry. The most reactive residue was always Tyr 411. Diethoxyphosphate-labeled Tyr 411 was stable for months at pH 7.4. These results will be useful in the development of specific antibodies to detect OP exposure, and to engineer albumin for use as an OP scavenger.

A PDF file of the published paper is attached.

Additional progress on Task 3. The following paper has been accepted for publication.

Covalent binding of the organophosphorus agent FP-biotin to tyrosine in eight proteins that have no active site serine

Grigoryan H, Li B, Anderson EK, Xue W, Nachon F, Lockridge O, Schopfer LM. Covalent binding of the organophosphorus agent FP-biotin to tyrosine in eight proteins that have no active site serine. Chem Biol Interact 2009 in press

ABSTRACT

Organophosphorus esters (OP) are known to bind covalently to the active site serine of enzymes in the serine hydrolase family. It was a surprise to find that proteins with no active site serine are also covalently modified by OP. The binding site in albumin, transferrin, and tubulin was identified as tyrosine. The goal of the present work was to determine whether binding to tyrosine is a general phenomenon. Fourteen proteins were treated with a biotin-tagged organophosphorus agent called FP-biotin. The proteins were digested with trypsin and the labeled peptides enriched by binding to monomeric avidin. Peptides were purified by HPLC and fragmented by collision induced dissociation in a tandem ion trap mass spectrometer. Eight proteins were labeled and six were not. Tyrosine was labeled in human alpha-2-glycoprotein 1 zinc-binding protein (Tyr 138, Tyr 174, Tyr 181), human kinesin 3C motor domain (Tyr 145), human keratin 1 (Tyr 230), bovine actin (Tyr 55, Tyr 200), murine ATP synthase beta (Tyr 431), murine adenine nucleotide translocase 1 (Tyr 81), bovine chymotrypsinogen (Tyr 201) and porcine pepsin (Tyr 310). Only 1-3 tyrosines per protein were modified, suggesting that the reactive tyrosine was activated by nearby residues that facilitated ionization of the hydroxyl group of tyrosine. These results suggest that OP binding to tyrosine is a general phenomenon. It is concluded that organophosphorus-reactive proteins include not only enzymes in the serine hydrolase family, but also proteins that have no active site serine. The recognition of a new OP-binding motif to tyrosine suggests new directions to search for mechanisms of long-term effects of OP exposure. Another application is in the search for biomarkers of organophosphorus agent exposure. Previous searches have been limited to serine hydrolases. Now proteins such as albumin and keratin can be considered.

A PDF file of the paper in press is attached.

Additional progress on Task 3. The following review summarizes our results for OP binding to tyrosine in proteins.

Mass Spectral Characterization of Organophosphate-Labeled, Tyrosine-Containing Peptides: Characteristic Mass Fragments and A New Binding Motif for Organophosphates

ABSTRACT

The target for organophosphates (OP) has traditionally been considered to be the active-site serine of serine esterases and proteases. Only recently has it become apparent that tyrosine, in peptides from a variety of proteins, represents another viable target for covalent modification by organophosphates. We have identified OP-tyrosine adducts on 12 different proteins (transferrin, serum albumin, kinesin 3C, alpha 2-glycoprotein 1 zinc, pro-apolipoprotein A-I, keratin, tubulin, actin, ATP synthase, adenine nucleotide Translocase I, chymotrypsinogen and pepsin), from 4 different species (human, cow, mouse and pig) labeled with 6 different OP (the chemical warfare agents soman and sarin, the pesticides chlorpyrifos-oxon and dichlorvos, and research compounds FP-biotin and diisopropylfluorophosphate). Labeling was achieved by treating pure proteins with up to a 40-fold molar excess of OP at pH 8-8.6. OP-treated proteins were digested with trypsin and peptides were separated by HPLC. Fragmentation patterns for 100 OP-peptides labeled on tyrosine were determined in the mass spectrometer. The goals of the present work were 1) to determine the common features of the OP-reactive tyrosines, and 2) to describe non-sequence MSMS fragments characteristic of OP-tyrosine peptides. These characteristic ions aid in identification of OP-tyrosine adducts. For substantial reaction to occur a mechanism for activating the tyrosine would be expected. Inspection of the crystal structures shows that in most cases there is a positively-charged amino acid (arginine, lysine or histidine) within 6 angstroms of the target tyrosine. This suggests that activation of the tyrosine for reaction with OP may involve through-space, charge-charge ion-pairing. Up to 7 tyrosines in albumin could be labeled with a 20-fold molar excess of OP, but only Tyr 411 reacted rapidly. Up to 16 tyrosines in tubulin could be labeled with a 20-fold molar excess of OP but only one tyrosine in beta tubulin was labeled with a low concentration of OP. Characteristic ions at 272 amu and 244 amu for tyrosine-OP immonium ions were nearly always present in the MSMS spectrum of peptides labeled on tyrosine by chlorpyrifos oxon. Characteristic fragments also appeared as a consequence of the neutral loss of OP side-chains from the parent ions that had been labeled with diisopropylfluorophosphate (216 amu), sarin (214 amu), soman (214 amu) or FP-biotin (227, 312, 329, 691 and 708 amu). In contrast to OP-reactive serines, which lie in the consensus sequence GXSXG, the OP-reactive tyrosines have no consensus sequence. Their common feature is the presence of nearby positively charged residues that activate the phenolic hydroxyl group. The significance of these findings is the recognition of a new binding motif for OP to proteins that have no active site serine. Modified peptides are difficult to find when the OP bears no radiolabel and no tag. The characteristic MSMS fragment ions we report are valuable because they are specific identifiers for OP-tyrosine, independent of the peptide.

INTRODUCTION

Organophosphates (OP) have long been known to react with enzymes of the serine esterase family (Schaffer et al., 1954; Main, 1979). The high reactivity of acetylcholinesterase [AChE, EC 3.1.1.7] with a variety of OP is the basis for the acute toxicity of these compounds (Maxwell et al., 2006). Covalent reaction of the OP with the catalytic serine of AChE inactivates the enzyme causing a myriad of cholinergic symptoms, including death (Brown and Brix, 1998). Other enzymes can be covalently labeled by OP (Casida and Quistad, 2004). Reaction of OP with some of these may explain non-cholinergic effects of OP.

Recent studies have demonstrated that OP can also react in vitro with tyrosine on a variety of proteins (Black et al., 1999; Schopfer et al., 2005; Li et al., 2007a; Grigoryan et al., 2008; Li et al., 2008; Li et al., 2009a). To date, we have found OP-tyrosine adducts on 12 different proteins (transferrin, serum albumin, kinesin 3C, alpha 2-glycoprotein 1 zinc, pro-apolipoprotein A-I, keratin, tubulin, actin, ATP synthase, adenine nucleotide Translocase I, chymotrypsinogen and pepsin), from 4 different species (human, cow, mouse and pig) labeled with 6 different OP (the chemical warfare agents soman and sarin, the pesticides chlorpyrifos-oxon and dichlorvos, and research compounds FP-biotin and diisopropylfluorophosphate).

Reaction of OP with tyrosine on serum albumin has been detected in vivo. Mice that were treated with 1 mg/kg of FP-biotin showed minimal signs of OP-toxicity, and still showed 1000-times more OP-labeled serum albumin than OP-labeled butyrylcholinesterase [BChE, EC 3.1.1.8] (Peeples et al., 2005). BChE is a classical, high-reactivity OP target. In another experiment, guinea pigs treated with either 0.5 LD_{50} amounts of sarin or 2-5 LD_{50} amounts of soman (together with medical countermeasures against intoxication) survived and were found to have substantial amounts of the OP bound to serum albumin (Williams et al., 2007).

The fact that a variety of proteins have been found to react with OP at tyrosine, coupled with the fact that this reaction can occur under relatively mild conditions in vivo, makes tyrosine an attractive candidate for a new, physiologically relevant target of OP intoxication.

To further explore the role of tyrosine as a physiological target for OP reaction, markers for the reaction products are of value.

Mass spectrometry is an excellent tool for identifying markers of protein modification. Three mass spectral features can be used for the identification. First, is the appearance of a mass in the MS spectrum of a protein that is consistent with a known peptide mass plus the mass of the modification. Second, is the presence of a gap in the b-ion or y-ion sequence from an MSMS spectrum that is consistent with the mass of a modified amino acid. And, third is the presence of fragments in the MSMS spectrum that are characteristic of the modification.

We have employed tandem quadrupole electrospray ionization mass spectrometry in conjunction with collision induced dissociation (CID) to study tyrosine-OP containing peptides. All three types of markers have been found. In all cases, the mass of the parent ion in the MS spectrum was consistent with the presence of the OP label. Manual analysis of the MSMS spectra from the labeled peptides often revealed gaps between b- and/or y-ions that were consistent with the mass of modified tyrosine. These gaps confirmed the presence of the OP and yielded the location of the labeled residue in the peptide sequence. Though each marker provides valuable information for the identification of modified peptides, the characteristic fragments are of particular value because they are specific identifiers for the type of modification, independent of the peptide.

The use of characteristic MSMS fragments for the detection of post-translational modifications was introduced by Huddleston for phosphorylation, glycosylation and sulfonylation (Huddleston et al., 1993). Subsequently, characteristic fragments for a wide variety of modifications have been identified. Characteristic fragments are frequently immonium ions, immonium ion-derived fragments or side chain fragments (Hung et al., 2007). Such fragments can be used for precursor ion scanning or for post-acquisition analysis using extracted ion chromatography. Additional fragments resulting from neutral loss reactions involving the modified amino acid are also common (Hung et al., 2007).

We have found sets of characteristic fragments for tyrosine adducts with soman, sarin, chlorpyrifos-oxon, dichlorvos, FP-biotin, and diisopropylfluorophosphate. Characteristic fragments include ions that are parts of the labeled tyrosine (immonium ions, immonium ion derived fragments); ions that appear as the result of neutral loss (neutral bits of the OP that are removed from the parent ion or sequence ions); and ions that there are unique fragments of the OP itself (found for FP-biotin).

This presentation has three goals. The first goal is to describe the characteristic ions for the various OP labels, along with the frequency at which each appears and the relative intensity of the signals. The second goal is to explore the environment of the labeled peptides in an attempt to establish factors that could promote the reaction of tyrosine with OP. We have found that tyrosines which are susceptible to reaction with OP frequently lie within 6 angstroms of a positively-charged group (lysine, arginine or histidine). This suggests that charge-charge, through-space ion-pairing may be lowering the pKa for these tyrosines, making them better nucleophiles–more capable of reacting with OP. The third goal is to establish tyrosine as a site for reaction of OP with proteins. This is consistent with the wide distribution of proteins that contain tyrosines which react with OP.

METHODS AND MATERIALS

The majority of the OP-labeled peptides reported in this article were taken from previously published reports, but a few were never before described. Preparation of the unreported peptides is described below.

Bovine actin was purchased from Sigma (St. Louis, MO, cat# 3653). It was dissolved in 130 µl of 10 mM ammonium bicarbonate, pH 8.3, to give a final concentration of 48 µM. Then it was incubated with 48, 240 or 2400 µM chlorpyrifos-oxon (CPO) at 37°C for 24 hours. The samples were boiled for 10 minutes to denature the protein then reduced with 10 mM dithiothreitol (Fisher Biotech, Fair Lawn, NJ, cat # BP172-25, electrophoresis grade), alkylated with 50 mM iodoacetamide (Sigma cat# I6125), and dialyzed against 4 liters of 10 mM ammonium bicarbonate, pH 8.3, for 18 hours with one change of buffer. It was digested with trypsin (porcine, sequencing grade modified trypsin cat# V5113, reductively methylated, TPCK treated from Promega, Madison, WI) at 37°C overnight. Trypsin was inactivated by boiling for 10 minutes. This preparation was used directly for MALDI mass spectrometry. A portion of the sample was dried in a vacuum centrifuge and redissolved in 5% acetonitrile/95% water/0.1% formic acid to yield approximately 3-5 pmole of peptide/µl (assuming no losses during processing) for analysis via electrospray-ionization, tandem triple-quadrupole mass spectrometry. Labeled peptides were identified by comparing the observed masses with the

expected masses for tryptic peptides from actin plus the added mass of CPO. Peptides identified in this manner were confirmed by manual analysis of the MSMS spectrum.

Human epidermal keratin was purchased from Sigma (St Louis, MO) (cat# K0253). It was supplied as a denatured mixture of keratins in 8 M urea plus 0.l mM beta-mercaptoethanol, and was subsequently renatured by dialysis against 25 mM Tris/Cl, pH 7.5 overnight. Then it was treated with 2 mM CPO and analyzed in the same manner as described for bovine actin.

Mouse tubulin beta appeared as a by-product during the analysis of a bovine tubulin tryptic digest. Refer to Grigoryan et al (2009a) for details of the preparation.

Human pro-apolipoprotein A-I was identified from a human serum sample. A 200 µl aliquot of serum was separated into high abundance and low abundance proteins using the Beckman Coulter Proteome IgY spin column depletion kit (Beckman Coulter, Fullerton, CA, cat# 24331). The high abundance fraction (240 µl containing 7.9 µg protein/µl) was incubated with 1.25 mM diisopropylfluorophosphate (DFP from Sigma cat# D0879) at 37°C overnight. The sample was denatured in 8 M urea, reduced with 5 mM DTT, alkylated with 40 mM iodoacetamide, dialyzed against 4 liters of 10 mM ammonium bicarbonate overnight (with two changes of buffer) and digested with trypsin (porcine, sequencing grade) at 37°C for 48 hours. The product, containing 4.4 µg protein/µl, was fractionated by strong cation exchange chromatography using a Polysulfoethyl A column (200 mm long x 2.1 mm diameter from PolyLC, Columbia, MD) on a Waters HPLC system (Waters, Milford, MA) with a 40 minute gradient starting at 100% solvent A and ending at 50% solvent A and 50% solvent B, where solvent A was 10 mM ammonium formate, pH 3.0, plus 25% acetonitrile and solvent B was 500 mM ammonium formate, pH 6.8, plus 25% acetonitrile. Fractions were dried in a vacuum centrifuge and resuspended in 5% acetonitrile/95% water/0.1% formic acid for analysis by electrospray-ionization triple-quadrupole mass spectrometry.

The preparation of the human serum samples from which the unassigned peptides were obtained was very similar to that used for pro-apolipoprotein A-I. The first major difference was that the serum was reacted with 200 µM FP-biotin for 48 hours at 37°C. Then after denaturation, reduction, alkylation, dialysis and tryptic digestion, the FP-biotinylated peptides were extracted using monomeric avidin-agarose beads (Pierce, Rockford, IL cat# 20228). The beads were washed with 0.5 M sodium chloride in 0.1 M Tris/Cl buffer, pH 8.6, followed by 20 mM Tris/Cl, pH 7.5. The FP-biotinylated peptides were eluted with 10% acetic acid. The eluate was dried in a vacuum centrifuge and resuspended in 5% acetonitrile/95% water/0.1% formic acid in preparation for analysis by electrospray-ionization triple-quadrupole mass spectrometry. Labeled peptides were identified by extracted ion chromatography utilizing the characteristic masses of 227, 312 and 329 amu derived from FP-biotin.

MALDI mass spectrometry. Generally, 1 microliter of tryptic digest (20-50 pmole/µl, assuming no losses during processing) was air dried onto a 384 well Opti-TOF sample plate (Applied Biosystems, Foster City, CA, #1016491) and then overlaid with 1 µl of alpha-cyano-4-hydroxy cinnamic acid solution (CHCA, 10 mg/ml from Fluka cat# 70990). CHCA was recrystallized before use then dissolved to 10mg/ml in 50% acetonitrile/50%water/0.1% trifluoroacetic acid. Mass spectra and collision induced MSMS spectra were collected in positive ion reflector mode with a MALDI TOF TOF 4800 mass spectrometer (Applied Biosystems). The final spectrum was the average of 500 laser shots. The mass spectrometer was calibrated before each use with CalMix 5 (Applied Biosystems).

Quadrupole mass spectrometry. Ten microliters of tryptic digest (30-50 pmole) were injected onto an HPLC nanocolumn (218MS3.07515 Vydac C18 polymeric reverse phase, 75 micron I.D. - 150 mm long; P.J. Cobert Assoc, St. Louis, MO). Peptides were separated with a 90 minute linear gradient from 5 to 60% acetonitrile at a flow rate of 0.3 μl/min and electrosprayed through a fused silica emitter (360 micron O.D., 75 micron I.D., 15 micron taper, New Objective, Woburn, MA) directly into the QTRAP 2000, a hybrid quadrupole linear ion trap mass spectrometer (Applied Biosystems). An ion-spray voltage of 1900 V was maintained between the emitter and the mass spectrometer. Information dependent acquisition was used to collect MS, high resolution MS, and MSMS spectra. All spectra were collected in the enhanced mode, using the trap function. The three most intense MS peaks in each cycle having masses between 200 and 1700 *m/z*, charge of +1 to +4, and intensities greater than 10,000 cps were selected for high resolution MS and MSMS analysis. Precursor ions were excluded for 30 s after one MSMS spectrum had been collected. MSMS fragmentation was obtained by collision induced dissociation (CID). The collision cell was pressurized to 40 μTorr with pure nitrogen. Collision energies between 20 and 40 eV were determined automatically by the software based on the mass and charge of the precursor ion. The mass spectrometer was calibrated on selected fragments from the MSMS spectrum of human Glu-fibrinopeptide B (Sigma cat# F3261).

Methodology. The discovery that tyrosines could be labeled by OP arose from a general search for proteins capable of reacting with OP. The strategy for the initial part of that search was taken from Cravatt and co-workers (Liu et al., 1999; Kidd et al., 2001), and employed the OP probe that they introduced (FP-biotin). One advantage of using FP-biotin as a probe is that the biotin tag allowed purification of the labeled protein. In general, the strategy consisted of the following: A crude protein preparation was labeled with 10 μM FP-biotin in pH 8.0 buffer. The labeled proteins were extracted from the crude mixture using avidin-agarose. The extracted proteins were separated by SDS PAGE. The presence of the FP-biotinylated proteins was confirmed by staining a blot of the PAGE gel with streptavidin-Alexa 680 (a fluorescent dye). Proteins present in sufficient quantity for mass spectral analysis were identified by staining a second PAGE gel with Coomassie Blue. Stained bands were cut from the gel and subjected to in-gel tryptic digestion. Finally, the tryptic digests were subjected to mass spectral analysis to identify the proteins that were present in the stained bands. This process is described in more detail by (Li et al., 2005; Peeples et al., 2005; Grigoryan et al., 2009b).

The primary difficulty with the search strategy described above is that the labeled peptides were not observed, so that proof for covalent binding of OP to proteins was indirect. Indirect evidence was acceptable for serine hydrolases, but not for proteins that had no active site serine. To find and characterize the labeled peptides, purified preparations of proteins that were identified in the initial screening were studied. This second-stage strategy consisted of the following: Purified proteins were labeled with FP-biotin. Labeled protein was proteolyzed. Labeled peptides were extracted with avidin-Agarose. Finally, the peptides were subjected to mass spectral analysis. Description of this process can be found in (Grigoryan et al., 2008; Li et al., 2009a). In another strategy a crude preparation of human plasma was labeled with FP-biotin, digested with trypsin and the labeled peptides extracted with monomeric avidin-agarose (Ding et al., 2008; Li et al., 2009a). With this process, labeled peptides could be successfully retrieved and characterized.

The third stage of the strategy tested the reactivity of pure proteins with OP that had no biotin tag. The OP-treated preparation was digested, and the peptides were analyzed mass spectrally. The masses of the known OP-reactive peptides plus the added mass from the new OP could be calculated and the MS spectrum searched for their presence. Characteristic ion masses provided means of searching through the MSMS data to locate peptides expected to become labeled, and to locate new peptides that might have become labeled. For most OP, the characteristic ion masses are too small and not sufficiently unique to serve as satisfactory search criteria for MSMS spectra from complex mixtures, unless a mass spectrometer of high mass accuracy is used, but they are fully adequate for searching MSMS spectra from pure protein digests. This means the protein of interest has to be purified from a complex mixture such as brain in order to find the OP-labeled peptide. Support for the conclusion that a peptide is labeled with OP on tyrosine comes from the presence in an MSMS spectrum of the characteristic OP-tyrosine immonium ions identified in this report.

RESULTS AND DISCUSSION

Distribution of OP labels. We found OP-labeled tyrosines on 60 different tryptic peptides. These peptides included 2 from human transferrin; 6 from human serum albumin; 1 from human kinesin KIF3C motor domain; 4 from unidentified proteins in human plasma; 3 from human alpha 2-glycoprotein 1, zinc; 1 from human pro-apolipoprotein A-I; 4 from human keratin 1; 1 from human keratin 2; 1 from human keratin 9; 2 from human keratin 10; 1 from bovine serum albumin; 9 from bovine tubulin alpha; 7 from bovine tubulin beta; 6 from bovine actin alpha, skeletal muscle; 1 from bovine chymotrypsinogen; 5 from mouse transferrin; 1 from mouse ATP synthase; 1 from mouse adenine nucleotide translocase I; 1 from mouse tubulin beta; 1 from pig pepsin and 2 synthetic peptides (See Table 3.1). There were 11 peptides that contained more than one tyrosine. Of these, 6 could be labeled on more than one tyrosine, and two of those (EEY*NGY*TGAFR and LY*LGHNY*VTAIR from mouse transferrin) could be labeled on two tyrosines simultaneously, though double labeling was not always observed.

Each labeled peptide exhibited the mass expected of the OP-adduct (to within 0.1 amu). The sequence of each peptide was confirmed by manual analysis of the CID MSMS spectrum. The location of the labeled amino acid could generally be established directly from the observed sequence. Finally, characteristic, non-sequence masses were identified that supported the proposed labeling.

Proteins for which we could find no FP-biotin-labeled peptides included porcine gelatin, bovine RNase, chicken lysozyme, bovine DNase I, human IgG and bovine insulin. Our inability to find OP-labeled peptides in these pure proteins treated with a 20-fold molar excess of FP-biotin does not rule out the possibility that these proteins can be labeled by OP. For example, hen egg white lysozyme incorporates 1 mole of DFP per mole of lysozyme (on tyrosine) at pH 9.5 when the DFP concentration is in 300-fold molar excess (Murachi et al., 1970). The OP-labeled lysozyme does not lose its esterase activity with p-nitrophenyl acetate.

Table 3.1. OP-Labeled Tyrosine-Containing Peptides Observed in Tryptic Digests

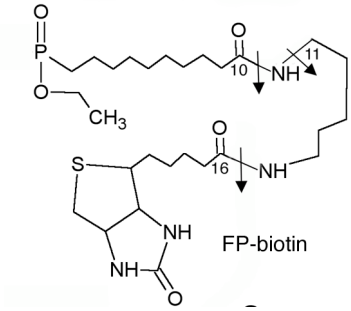
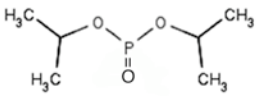
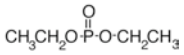
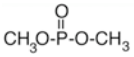
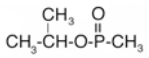
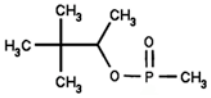
#	Species	Protein	Peptide [a]	gi: number [c]	OP [d]	reference
1	Human	Transferrin	KPVDEY*K	136191	FPB, DFP, DCV, CPO, soman	(Li et al., 2009a)
2	Human	Transferrin	KPVEEY*ANCHLAR	136191	FPB, DFP, CPO, soman, sarin	(Li et al., 2009a)
3	Human	Serum Albumin	Y*TK	28592	FPB, CPO, DFP	(Li et al., 2007a; Williams et al., 2007; Ding et al., 2008)
4	Human	Serum Albumin	HPY*FY*APELLFFAK	28592	FPB, CPO	(Ding et al., 2008)
5	Human	Serum Albumin	Y*LYEIAR	28592	FPB, CPO	(Ding et al., 2008)
6	Human	Serum Albumin	QNCELFEQLGEY*K	28592	FPB, CPO	(Ding et al., 2008)
7	Human	Serum Albumin	MPCAEDY*LSVVLNQLCLHEK	28592	FPB, CPO	(Ding et al., 2008)
8	Human	Serum Albumin	Y*KAAFTECCQAADK	28592	CPO	(Ding et al., 2008)
9	Human	Kinesin 3C motor domain 5	ASYY*LEIYQEEIR	41352705	FPB	(Grigoryan et al., 2009b)
10	Human	Serum Unknown	AY*PR	-	FPB	this work
11	Human	Serum Unknown	Y*PR	-	FPB	this work
12	Human	Serum Unknown	Y*L/IK	-	FPB	this work
13	Human	Serum Unknown	Y*K	-	FPB	this work
14	Human	a2-Glycoprotein 1, zinc	WEAEPVY*VQR	51094610	FPB	(Grigoryan et al., 2009b)
15	Human	a2-Glycoprotein 1, zinc	AY*LEEECPATLR	51094610	FPB	(Grigoryan et al., 2009b)
16	Human	a2-Glycoprotein 1, zinc	YY*YDGKDYIEFNK [b]	51094610	FPB	(Grigoryan et al., 2009b)
17	Human	Pro-apolipoprotein A-I	DY*VSQFEGSALGK	178775	DFP	this work
18	Human	Keratin 1 renatured	LLRDY*QELMNTK	119395750	CPO	this work
19	Human	Keratin 1 renatured	SGGGFSSGSAGIINY*QR	119395750	CPO	this work
20	Human	Keratin 1 renatured	THLEPY*FESFINNLR	119395750	FPB, CPO	Erica paper
21	Human	Keratin 1 renatured	Y*EELQITAR	119395750	CPO	this work
22	Human	Keratin 2 renatured	Y*LDGLTAER	181402	CPO	this work
23	Human	Keratin 9 renatured	QFSSSY*LSR	435476	CPO	this work
24	Human	Keratin 10 renatured	LKY*ENEVALR	47744568	CPO	this work
25	Human	Keratin 10 renatured	LASY*LDK	47744568	CPO	this work
26	Bovine	Serum albumin	Y*TR	30794280	FPB, CPO, DFP	(Schopfer et al., 2005)
27	Bovine	Tubulin alpha	TGTY*R	73586894	FPB, DFP, CPO	(Grigoryan et al., 2008)
28	Bovine	Tubulin alpha	AFVHWY*VGEGMEEGEFSEAR	73586894	CPO	(Grigoryan et al., 2009a)
29	Bovine	Tubulin alpha	EDAANNY*AR	73586894	CPO	(Grigoryan et al., 2009a)
30	Bovine	Tubulin alpha	IHFPLATY*APVISAEK	73586894	CPO	(Grigoryan et al., 2009a)
31	Bovine	Tubulin alpha	FDGALNVDLTEFQTNLVPY*PR	73586894	CPO	(Grigoryan et al., 2009a)
32	Bovine	Tubulin alpha	GHY*TIGK	73586894	CPO	(Grigoryan et al., 2009a)

33	Bovine	Tubulin alpha	VGINY*QPPTVVPGGDLAK	73586894	CPO	(Grigoryan et al., 2009a)
34	Bovine	Tubulin alpha	FDLMY*AK	73586894	CPO	(Grigoryan et al., 2009a)
35	Bovine	Tubulin alpha	LSVDY*GK	73586894	CPO	
36	Bovine	Tubulin beta	Y*VPR	75773583	FPB, DFP, CPO, soman	(Grigoryan et al., 2008)
37	Bovine	Tubulin beta	GSQQY*R	75773583	FPB, DFP, CPO, sarin, soman	(Grigoryan et al., 2008)
38	Bovine	Tubulin beta	EEY*PDR	75773583	FPB, DFP CPO	(Grigoryan et al., 2008)
39	Bovine	Tubulin beta	Y*LTVAAVFR	75773583	CPO	(Grigoryan et al., 2009a)
40	Bovine	Tubulin beta	GHY*TEGAELVDSVLDVVR	75773583	CPO	(Grigoryan et al., 2009a)
41	Bovine	Tubulin beta	INVY*Y*NEATGGK	75773583	CPO	(Grigoryan et al., 2009a)
42	Bovine	Tubulin beta	NSSY*FVEWIPNNVK	75773583	CPO	(Grigoryan et al., 2009a)
43	Bovine	Actin alpha skeletal muscle	GY*SFVTTAER	62287933	FPB, CPO	(Grigoryan et al., 2009b) this work
44	Bovine	Actin alpha skeletal muscle	DSY*VGDEAQSK	62287933	FPB	(Grigoryan et al., 2009b)
45	Bovine	Actin alpha skeletal muscle	SY*ELPDGQVITIGNER	62287933	CPO	this work
46	Bovine	Actin alpha skeletal muscle	IWHHTFY*NELR	62287933	CPO	this work
47	Bovine	Actin alpha skeletal muscle	QEY*DEAGPSIVHR	62287933	CPO	this work
48	Bovine	Actin alpha skeletal muscle	DLTDY*LMK	62287933	CPO	this work
49	Bovine	Chymotrypsinogen	Y*TNANTPDR	194674931	FPB	(Grigoryan et al., 2009b)
50	Mouse	Transferrin	KPVDQY*EDCY*LAR	21363012	FPB, DFP CPO, sarin soman	(Li et al., 2009a)
51	Mouse	Transferrin	LY*LGHNY*VTAIR	21363012	FPB, CPO	(Li et al., 2009a)
52	Mouse	Transferrin	EEY*NGY*TGAFR	21363012	FPB, CPO	(Li et al., 2009a)
53	Mouse	Transferrin	FDEFFSQGCAPGY*EK	21363012	FPB, CPO	(Li et al., 2009a)
54	Mouse	Transferrin	GY*Y*AVAVVK	21363012	FPB, CPO, DFP, sarin, soman	(Li et al., 2009a)
55	Mouse	ATP Synthase	ILQDY*K	20455479	FPB	(Grigoryan et al., 2009b)
56	Mouse	Adenine Nucleotide Translocase I	Y*FPTQALNFAFK	902008	FPB	(Grigoryan et al., 2009b)
57	Mouse	Tubulin beta	INVY*Y*NEAAGNK	21746161	CPO	this work
58	Porcine	Pepsin	QYY*TVFDR	1302650	FPB	(Grigoryan et al., 2009b)
59	Synthetic	peptide	RY*TR		CPO	(Li et al., 2009a)
60	Synthetic	peptide	SY*SM		DCV	(Li et al., 2009a)

a. The asterisk on Tyrosine (Y*) indicates the labeled tyrosine. For peptides showing two Y*, either one or the other tyrosine was labeled.
b. The position of the label is unclear for this peptide. Either the first or second Y from the N-terminus could have been labeled.
c. The gi number is the NCBI accession number for the protein in PubMed.
d. FPB= FP-biotin; CPO= chlorpyrifos oxon; DFP=diisopropyl fluorophosphate; DCV=dichlorvos

OP Characteristic Fragments. We employed 6 different organophosphorus agents in these studies: soman and sarin (chemical warfare agents), chlorpyrifos-oxon and dichlorvos (commercial pesticides), and FP-biotin and diisopropylfluorophosphate (research reagents). Table 3.2 gives the OP structures and their added masses, after the OP have made a covalent bond with tyrosine.

Table 3.2. Structures of Organophosphorus Agents Bound to Tyrosine

Structure of the added mass	Added Mass (amu)	Organophosphorus Agent
	572	FP-biotin
	164	Diisopropylfluorophosphate (DFP)
	136	Chlorpyrifos-oxon (CPO)
	108	Dichlorvos (DCV)
	120	Sarin
	162	Soman

The arrows in the FP-biotin structure indicate fragmentation sites. A 227 amu ion is produced by cleavage between carbon 16 and the adjacent nitrogen. A 329 amu ion is produced by cleavage between carbon 10 and the adjacent nitrogen. The 312 amu ion is produced by loss of amine from the 329 amu ion.
Added mass refers to the mass of the OP that remains covalently attached to the target amino acid after the reaction is complete.

The reaction between OP and tyrosine is illustrated in Figure 3.1. Soman makes a covalent bond with the phenolic oxygen of tyrosine and simultaneously releases fluoride. The reaction product has an added mass of 162 amu. The OP-tyrosine adduct is stable and does not undergo the dealkylation reaction called "aging" that is typical of soman adducts on acetylcholinesterase.

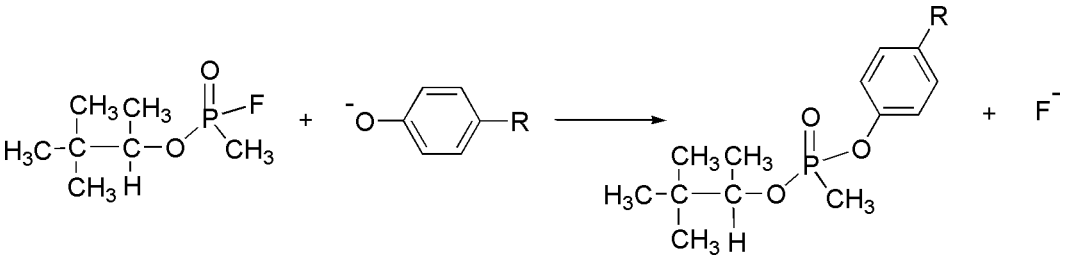


Figure 3.1. Modification of tyrosine by soman to make a stable covalent bond.

Many of the 60 peptides described in Table 3.1 were labeled with more than one OP. Consequently, a total of 100 variously-labeled peptides could be assembled: 6 labeled with soman, 4 with sarin, 45 with chlorpyrifos-oxon, 2 with dichlorvos, 32 with FP-biotin, and 11 with diisopropylfluorophosphate. Each peptide was labeled on tyrosine. Each type of labeled tyrosine generated characteristic non-sequence ions upon low-energy, collision induced dissociation (CID) in the mass spectrometer (QTRAP 2000, Applied Biosystems). Collision energies were set at 20-60 eV and nitrogen was used as the collision gas, at $4x10^{-5}$ Torr.

The non-sequence fragments can be divided into three categories: 1) those that arise by neutral loss of a side-chain from an OP-tyrosine adduct of the parent ion or a fragment ion, 2) those that are OP-tyrosine immonium ions or derivatives of OP-tyrosine immonium ions, and 3) those that arise by fragmentation that is specific to FP-biotin.

Neutral loss of a side-chain from CPO, DFP, soman and sarin adducts. In the context of these experiments, neutral loss from CPO, DFP, soman and sarin refers to the loss of an alkyl side-chain from an OP-tyrosine adduct. It does not refer to loss of the entire OP from the tyrosine. None of the 100 OP-tyrosine peptide MSMS spectra we examined showed loss of the entire OP from tyrosine. In contrast, phosphorylated-tyrosine cab lose the entire phosphate, though in low yield (Tholey et al., 1999).

The neutral loss of alkyl side-chains from OP in the gas phase can be attributed to McLafferty rearrangement (McLafferty, 1959). This rearrangement (Figure 3.2) requires the availability of a proton on a carbon atom beta to the phosphorus oxygen. Loss of the alkyl group occurs with concomitant transfer of the beta-proton to the phosphyl-oxygen, via a six-membered transition state, and formation of a carbon-carbon double bond between the alpha and beta carbons in the alkyl group. Under low-energy CID conditions, this is a facile reaction.

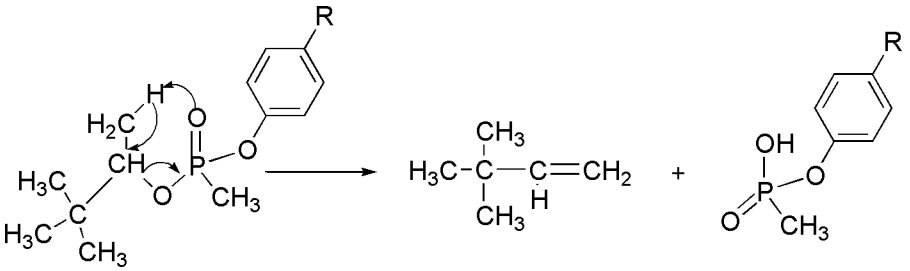


Figure 3.2. McLafferty rearrangement of a tyrosyl-soman adduct resulting in neutral loss of the pinacolyl side chain. The pinacolyl side-chain is lost during MSMS under CID conditions. Soman-tyrosine adducts do not lose the pinacolyl group outside of the mass spectrometer.

McLafferty rearrangement is sometimes referred to as beta-elimination, by analogy with the hydroxide-catalyzed elimination of phosphate from phosphoserine and phosphothreonine in solution chemistry. McLafferty rearrangement/beta elimination is responsible for the well-documented release of phosphate from phosphoserine and phosphothreonine during CID (Tholey et al., 1999). This mechanism also provides the primary justification for why release of phosphate (or organophosphate) from phosphotyrosine is chemically unfavorable, i.e. the beta proton in phosphotyrosine is on an aromatic ring and therefore not readily released.

Most of the alkoxy side-chains on the OP given in Table 3.2 are susceptible to McLafferty rearrangement. Methoxy derivatives, such as are found in dichlorvos, do not have a beta-carbon and therefore are not susceptible to McLafferty rearrangement. Alkyl ligands that attach directly to the phosphorus, without the intervention of an oxygen molecule, are also not susceptible to McLafferty rearrangement, i.e. phosphonyl esters. Ligands of this latter type are found on soman and sarin (methyl) and FP-biotin (biotinylated arm).

McLafferty rearrangements generate neutral losses of 28 amu for ethoxy side-chains (CPO and FP-biotin), 42 amu for isopropoxy side-chains (DFP and sarin), and 84 amu for the pinacolyl side-chain of soman.

Neutral loss of OP side-chains was observed in two contexts. In the first context, loss was directly from the parent ion. This could be detected if the peptide was small enough for the diminished parent ion mass to fall within the mass range of the QTrap 2000 mass spectrometer (upper limit 1500 m/z). An example of this sort of neutral loss is shown in Figure 3.3 where a doubly-charged form of the parent ion minus the pinacolyl side-chain of the soman label can be found at 524.2 amu. A second example is found in Figure 3.6 where the singly-charged, DFP-labeled, YTR parent ion (603.4 amu) loses one isopropyl group (42 amu) to yield the 561.4 ion, and then a second isopropyl group (a total of 84 amu) to yield the 519.2 ion.

In the second context, neutral loss was from OP-labeled tyrosine immonium ions. Loss from immonium ions was a common occurrence and is readily observable in several of the following figures.

The order of neutral loss susceptibility observed for peptides labeled with the various OP, correlated with the complexity of the alkyl chain. Thus, after CID fragmentation of soman-labeled peptides, ions carrying intact soman labels were never observed. Only masses associated with ions missing the pinacolyl group were found (see Figure 3.3). On the other hand, the MSMS spectra of DFP-labeled peptides showed ions carrying the intact DFP label as well as

ions that had lost one or both of the isopropyl groups (see Figure 3.6). This distribution reflects the decreased susceptibility of the isopropoxy group to undergo neutral loss. Finally, the MSMS spectra of CPO-labeled peptides rarely showed parent ion masses consistent with the loss of an ethyl group. Loss of the ethyl group was only seen from the labeled tyrosine immonium ion which created a tyrosine-monoethylphosphate immonium ion at 244.0 amu (see Figure 3.5). Neutral loss of methyl was not detected from DCV-labeled peptides (see Figure 3.7). Side-chain neutral losses have also been reported for the isolated OP, e.g. sarin, soman, cyclosarin and analogs of DFP and CPO (Bell et al., 1997; D'Agostino et al., 2006).

A similar loss of the alkyl side-chain occurs when OP are bound to cholinesterases in solution. This process is referred to as aging. The gas phase side-chain loss is mechanistically distinct from the aging process and should not be confused with aging.

The observations on neutral loss can be summarized by saying that loss of a side-chain from alkoxy ligands to the phosphorus was the rule for soman-labeled tyrosine; was common for DFP- or sarin-labeled tyrosine; was constrained to tyrosine immonium ions for CPO-labeled tyrosine; and was not observed for the dichlorvos-labeled tyrosine.

OP-tyrosine immonium ions. With the exception of FP-biotinylated samples, the non-sequence masses that appeared most frequently were consistent with forms of OP-tyrosine immonium ions. This is in agreement with a recent review by Lehmann and coworkers, in which they reported that CID-generated characteristic ions (reporter ions) for peptides containing covalently modified amino acids are generally immonium ions or immonium ion-derived fragments (Hung et al., 2007). The OP-tyrosine immonium ions and their derivatives appear in the list of characteristic ions in Table 3.3.

Two dichlorvos-labeled tryptic peptides were analyzed. A mass at 244 amu, consistent with the dimethoxyphospho-tyrosine immonium ion, was the most prominent peak in both MSMS spectra (see Figure 3.7 for an example). This mass did not contribute to the peptide sequence information. Though the 244 amu mass is identical to the mass for phospho-tyrosine, the anticipated difficulty in releasing methyl groups from methoxy-OP makes an immonium ion of dimethoxyphospho-tyrosine the more reasonable assignment.

The diethoxyphospho-tyrosine immonium ion, at 272 amu, was the most commonly observed non-sequence ion for chlorpyrifos-oxon labeled peptides. It appeared in 92% of the MSMS spectra (42 out of 45 peptides). In addition to being common, the mass was generally intense. It was the most intense ion in 7 spectra. The monoethoxyphospho-tyrosine immonium ion, at 244 amu, was nearly as abundant as the diethoxyphospho-tyrosine immonium ion, appearing in 75% of the spectra (34 out of 45 peptides). It was also intense, being the most intense ion in 2 spectra. For an example, refer to Figure 3.5. Though the 244 amu mass that we have taken to be the monoethoxyphospho-tyrosine immonium ion is the same as that for phospho-tyrosine, the prevalence of immonium ions in Table 3.3 makes the immonium ion assignment for this mass the more reasonable. Elimination of both ethyl groups to yield the phospho-tyrosine immonium ion, at a mass of 216 amu, was less common, occurring in 30% of the spectra (14 out of 45). The FP-biotin tyrosine adduct also includes an ethoxy substituent on the phosphorus, but no evidence for an FP-biotin tyrosine immonium ion with loss of the ethyl was detected.

By far, the most common non-sequence ion seen in the spectra of the DFP-labeled peptides was the 216 amu mass attributable to the phospho-tyrosine immonium ion, i.e. loss of both isopropyl groups (see Table 3.3 and Figure 3.6). It appeared in 91% of the MSMS spectra

(10 out of 11 peptides). Though its intensity varied widely, it was always at least 20% that of the most intense peak in the spectrum. On one occasion it was the most intense peak in the spectrum. Loss of a single isopropyl to generate the monoisopropoxyphospho-tyrosine immonium ion at 258 amu appeared in only 9% of the spectra (1 out of 11 peptides). The preponderance of species in which both isopropyl groups had been lost reflects the ease of eliminating the isopropyl under CID conditions. The next most common characteristic ion was the phospho-tyrosine immonium ion minus water at 198 amu, occurring in 27% of the spectra (3 out of 11). This species is a secondary fragmentation of the phospho-tyrosine immonium ion and supports the dominance of the isopropyl free form.

Tyrosine immonium ions from the four sarin-labeled peptides that were examined all had lost their isopropyl moieties (Figure 3.8). The resultant methylphospho-tyrosine immonium ion at 214 amu was the only non-sequence ion seen in the MSMS spectra, and it was always the most intense ion in the spectrum (see Table 3.3).

Formation of the methylphospho-tyrosine immonium ion from soman-labeled tyrosine containing peptides was also commonplace, appearing in 83% of the MSMS spectra. However, when it did occur, the relative intensity of the peak was generally less than 20% that of the most intense peak in the spectrum (see Table 3.3 and Figure 3.3). By extrapolation of the trend developed from the smaller alkoxy ligands, one might have expected that this immonium ion would have been present for all soman-labeled peptides and that it would have dominated the spectra. A possible rationalization for the unexpected loss in prominence of the methylphospho-tyrosine immonium ion may lie in the extremely facile nature of the neutral loss of pinacolyl from the parent ion. In the same manner that facile loss of phosphate from the parent ions of phospho-peptides suppresses other fragmentation (Beck et al., 2003; Steen et al., 2006), facile loss of the pinacolyl group from the soman-labeled parent ions may suppress formation of the methylphospho-tyrosine immonium ion.

The FP-biotinylated tyrosine immonium ion, at 708.5 amu, appeared in 90% of the fragmentation spectra for FP-biotin-labeled tyrosine-containing peptides. It was generally accompanied by a 691.4 amu mass that was consistent with the loss of amine from the tyrosine-FP-biotin immonium ion (see Figure 3.4). Even the doubly-charged forms of these ions, at 355.0 and 346.5 amu were common, appearing in 40 and 17% of the spectra, respectively (see Table 3.3).

Observations on the OP-tyrosine immonium ions can be summarized by saying that they are major non-sequence ions in the MS/MS spectra, and that they are characteristic of OP-labeling of tyrosine.

Fragmentation specific to FP-biotin

In addition to OP-tyrosine immonium ions, fragmentation of FP-biotin labeled tyrosine yielded a unique set of ions. Elimination of the ethyl group from the ethoxy side-chain of the phosphorus was not seen. Rather, fragmentation of the amide linkages in the biotinyl side-chain of the FP-biotin generally dominated the MSMS spectrum. See Table 3.3 for a list of these fragments and Table 3.2 for a graphical presentation of the location of the break points.

An FP-biotinylated peptide is invariably multiply-charged. This appears to be due to protonation of the biotin. Thus, even two and three residue peptides are doubly-charged. Scission of FP-biotin's alkyl chain releases the biotin end of the label, along with one charge. This generates positively-charged fragments, as well as creating a loss from the parent ion. A hallmark of FP-biotinylation on tyrosine is the presence of positively-charged fragments at 227,

312 and 329 amu (Schopfer et al., 2005), see Figure 3.4. Fragments at 329 and 312 amu appeared in 100% of the spectra, while fragments at 227 amu appeared in 89% (31 out of 35 peptides). All three fragments generally yielded very intense signals (see Table 3.3). The 329 amu mass was the most intense peak on 6 occasions, the 312 amu peak on 2 occasions, and the 227 amu peak on 3.

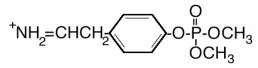
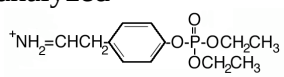
A corresponding neutral loss of 226, 311 and 328 amu from the parent ion was detected in 34%, 11% and 31% of the MSMS spectra, respectively. The 328 amu neutral loss was always accompanied by a 329 amu loss. The parent ion minus 329 could be discriminated from the +1 isotopic form of the parent ion minus 328 on the basis of the relative intensities of the [M+H-328] and [M+H-329] peaks. The intensities of these two masses were always nearly equal.

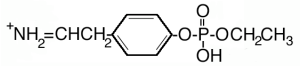
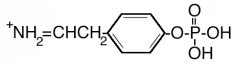
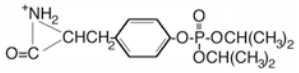
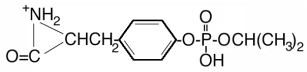
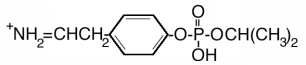
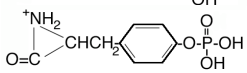
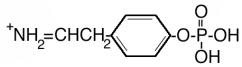
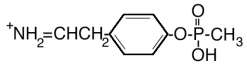
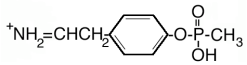
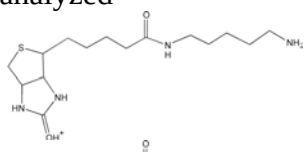
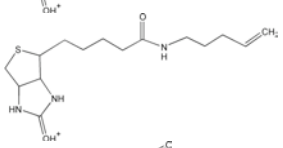
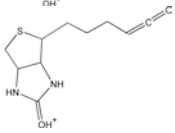
Neutral loss directly from the parent ion was not observed as frequently as the corresponding positively-charged fragment ion. The parent ion minus 328 (and 329) appeared in 31% of the spectra (11 out of 35); the parent ion minus 311 appeared in 11% of the spectra (4 out of 35); and the parent ion minus 226 appeared in 34% of the spectral (12 out of 35). This can be attributed in part to the large size of the FP-biotin adduct, which has a 572 amu added mass. Since the upper mass limit for the QTrap 2000 is about1500 amu, neutral loss fragments from FP-biotinylated peptides larger than 7 residues were difficult to detect. Neutral loss fragments were occasionally accompanied by fragments consistent with loss of water or amine or CO.

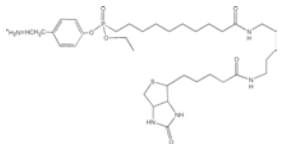
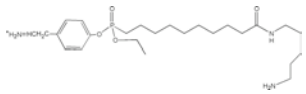
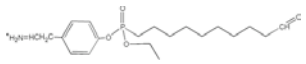
Doubly-charged fragments corresponding to the parent ion minus 328, 329 or 226 amu were also detected, as well as fragments corresponding to loss of amine from these species. In addition, tyrosine-FPB immonium ions that had lost 329 amu to give a mass of 379 amu (17%) or 328 amu to give a mass of 380 amu (40%), 226 amu to give a mass of 482 amu (31%) were commonly seen (Table 3.3).

Ions at 227, 312 and 329 amu generally appear when either FP-biotinylated tyrosine or serine adducts are fragmented (Schopfer et al., 2005). These three ions provide a fingerprint for FP-biotinylated peptides. Since they can be relied upon to appear as a triad and they have moderately large molecular weights, confusing this set of masses with sequence masses from the peptide is minimal. Discrimination between FP-biotinylated tyrosine and FP-biotinylated serine can also be accomplished on the basis of characteristic masses. The FP-biotinylated tyrosine immonium ions at 708 and 691 amu provide a diagnostic sub-set of masses that identify FP-biotinylated tyrosine, while a fragment at 591 amu is characteristic of FP-biotinylated serine (Schopfer et al., 2005). This array of characteristic masses makes FP-biotin very useful in the discovery of unknown proteins that are susceptible to labeling by OP.

Table 3.3. OP-Tyrosine Characteristic Ions [a]

Mass amu [b]	Name	Structure	Per Cent [c]	Rel Intense [d]
	Dichlorvos 2 tryptic peptide analyzed			
244.0	dimethoxyphospho Tyr immonium		100	100
	Chlorpyrifos-oxon 45 tryptic peptides analyzed			
272.3	diethoxyphospho Tyr immonium		92	6-100

253.5	diethoxyphospho Tyr immonium -NH_3		5	4
254.2	diethoxyphospho Tyr immonium -H_2O		2	4
244.3	monoethoxyphospho Tyr immonium		75	30-100
226.1	monoethoxyphospho Tyr immonium -H_2O		16	2-38
216.1	phospho Tyr immonium		30	3-77
198.1	phospho Tyr immonium -H_2O		2	16
	Diisopropylfluorophosphate			
	11 tryptic peptides analyzed			
328.8	diisopropoxyphospho Tyr		9	23
311.8	diisopropoxyphospho Tyr -NH_3		9	9
286.2	monoisopropoxyphospho Tyr		9	6
258.1	monoisopropoxyphospho Tyr immonium		9	4
244.2	phospho Tyr		9	60
226.0	phospho Tyr -H_2O		18	8-100
216.1	phospho Tyr immonium		91	4-100
199.2	phospho Tyr immonium - NH_3		9	24
198.1	phospho Tyr immonium -H_2O		27	6-22
	Sarin			
	4 tryptic peptides analyzed			
214.1	methylphospho Tyr immonium		100	100
	Soman			
	6 tryptic peptides analyzed			
214.2	methylphospho Tyr immonium		83	10-95
	FP-Biotin			
	32 tryptic peptides analyzed			
329.4	FPB fragment		100	6-100
312.4	FPB fragment		100	7-100
227.2	FPB fragment		89	4-100

708.5	Tyr-FPB immonium		86	5-100
691.4	Tyr-FPB immonium -NH_3		86	5-80
482.5	Tyr-(FPB-226) immonium		31	10-15
465.5	Tyr-(FPB-226) immonium-NH_3		20	5-20
397.0	Tyr-(FPB-311) immonium		3	14
380.4	Tyr-(FPB-328) immonium		40	15-25
379.3	Tyr-(FPB-329) immonium		17	10-20
362.8	Tyr-(FPB-328) immonium-NH_3		3	27
355.0	Tyr-FPB immonium (+2 charge)		43	2-100
346.5	Tyr-FPB immonium -NH_3 (+2 charge)		20	10-35

a All masses are for the protonated, dehydro form of the amino acid.
b Masses are given as the average of all measurements made from the quadrupole tandem mass spectrometer.
c "Per Cent" refers to the fraction of the tryptic peptides that exhibited this mass.
d "Rel Intense" is given as a range, which refers to the intensity of the mass relative to the most intense peak in the MSMS spectrum. This value is given as a percentage. Entries without a range represent masses which appeared only once, except for the 214.1 amu entry for sarin which appeared for all 3 peptides as the most intense peak of the spectrum.

Illustration of CID Fragmentation for OP-Labeled Peptides. Figures 3.3 through 3.8 show representative MSMS fragmentation spectra for peptides containing OP-labeled tyrosine. There is a spectrum for each of the six OP that were used in these studies. Fragmentation spectra from other OP-labeled, tyrosine-containing peptides can be found in the literature (Schopfer et al., 2005; Li et al., 2007a; Ding et al., 2008; Grigoryan et al., 2008; Li et al., 2008).

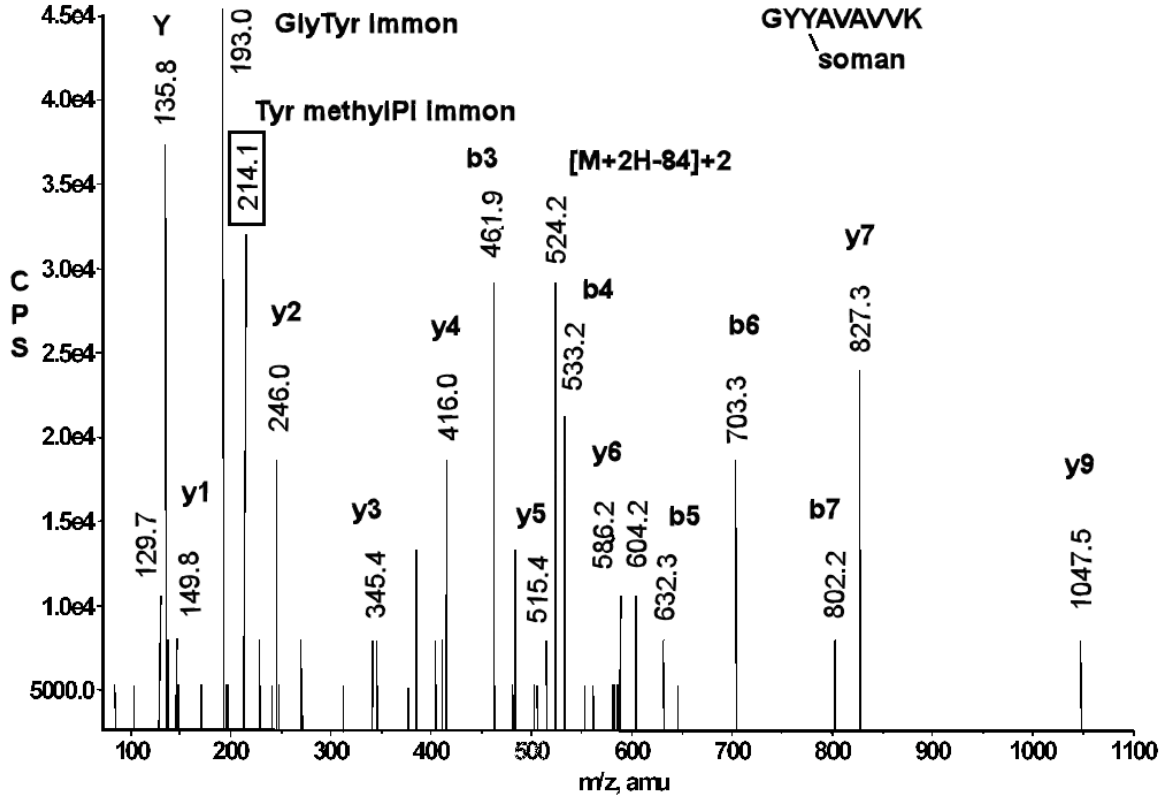


Figure 3.3. A CID fragmentation spectrum of the soman-labeled mouse transferrin, tryptic peptide GYY*AVAVVK. The value enclosed in the box is the mass of the characteristic fragment for soman-labeled tyrosine. MethylPi stands for methylphosphonate.

Figure 3.3 illustrates the CID fragmentation of a soman-labeled peptide. This peptide is GYY*AVAVVK from mouse transferrin, which is labeled on the second tyrosine from the N-terminus. The parent ion was doubly-charged with an m/z of 566.5 that includes an added mass of 162 amu from soman (O-pinacolyl methylphosphonate). Fragment y9 is consistent with the singly-charged parent ion less the mass of the pinacolyl side-chain (84 amu). This neutral loss leaves a methylphosphonate moiety attached to the peptide fragment (78 amu). The doubly-charged methylphosphonate parent ion was found at 524.2 amu. No fragment mass that included the O-pinacolyl methylphosphonate was detected, reflecting the extreme ease with which the pinacolyl group is eliminated under CID conditions. Fragments y1 to y9 represent the entire y-ion series, wanting only y8. Fragments y7 and y9 include the mass for the methylphosphonate as expected. The mass difference of 241.1 amu between y6 and y7 is consistent with tyrosine plus methylphosphonate (163.1 + 78 amu). A b-ion series from b3 to b7 is also present. The b3 mass, at 461.9 amu, and all subsequent b-ion masses include the mass of methylphosphonate. A tyrosine-glycine immonium ion at 193.0 amu does not include the methylphosphonate mass, indicating that the first tyrosine from the N-terminal is not labeled. This observation together with fact that the b3-ion includes the methylphosphonate places the soman label on the second tyrosine from the N-terminal. The characteristic tyrosine methylphosphonate immonium ion appears at 214.1 amu, confirming the presence of soman on the original peptide.

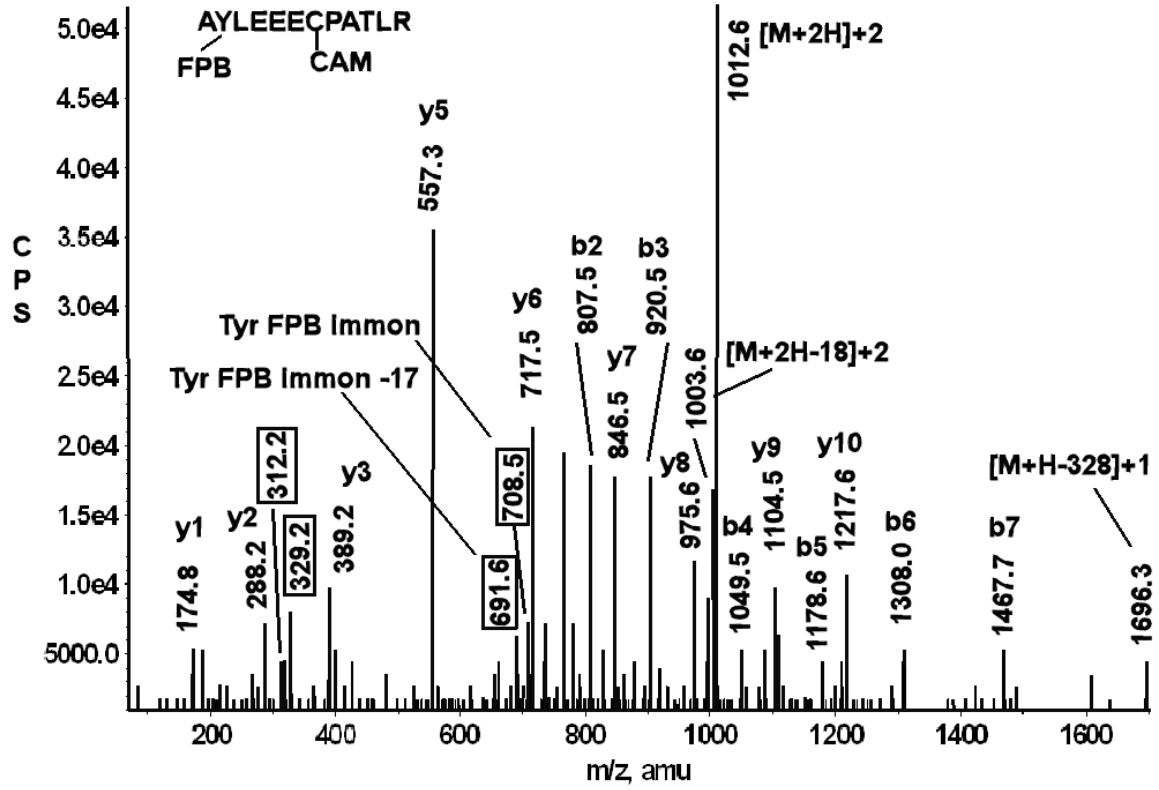


Figure 3.4. A CID fragmentation spectrum of the FP-biotin labeled human alpha 2 glycoprotein 1, zinc tryptic peptide AY*LEEECPATLR. The values enclosed in the boxes are the masses of the characteristic fragments for FP-biotin labeled tyrosine. CAM indicates that the cysteine is carbamidomethylated.

Figure 3.4 illustrates the CID fragmentation of an FP-biotin labeled peptide. This peptide is AY*LEEECPATLR from human alpha 2 glycoprotein 1, zinc, which is labeled on the second residue from the N-terminus. The parent ion is doubly-charged with an m/z of 1012.7 that includes the 572 amu added mass from FP-biotin (10-(ethoxyphosphinyl)-N-(biotinamidopentyl) decanamide minus a proton) and a carbamidomethyl modification on cysteine (57 amu). Another doubly-charged ion at 1003.6 amu is consistent with the parent ion minus water. A fragment at 1696.3 amu represents the neutral loss of a 328 amu portion of FP-biotin from the parent ion. A y-ion series from y1 to y10 is observed. The masses of all of these fragments fit to the unlabeled sequence. A b-ion series from b2 to b7 is also observed. The b2 mass (807.5 amu) is consistent with the presence of alanine, tyrosine and the FP-biotin label (164 + 71 + 572 = 807 amu). Successive b-ion masses all include the mass of the FP-biotin label. Carbamidomethyl Cys (CAM, cysteine plus 57 amu) appears in both the y6 and y7 ions. Characteristic fragments of FP-biotin appear at 312.2 and 329.2 amu, while immonium ions for tyrosine FP-biotin and tyrosine FP-biotin minus amine appear at 708.5 and 691.6 amu, respectively. These are four of the most commonly observed characteristic ions from FP-biotin labeled, tyrosine-containing peptides.

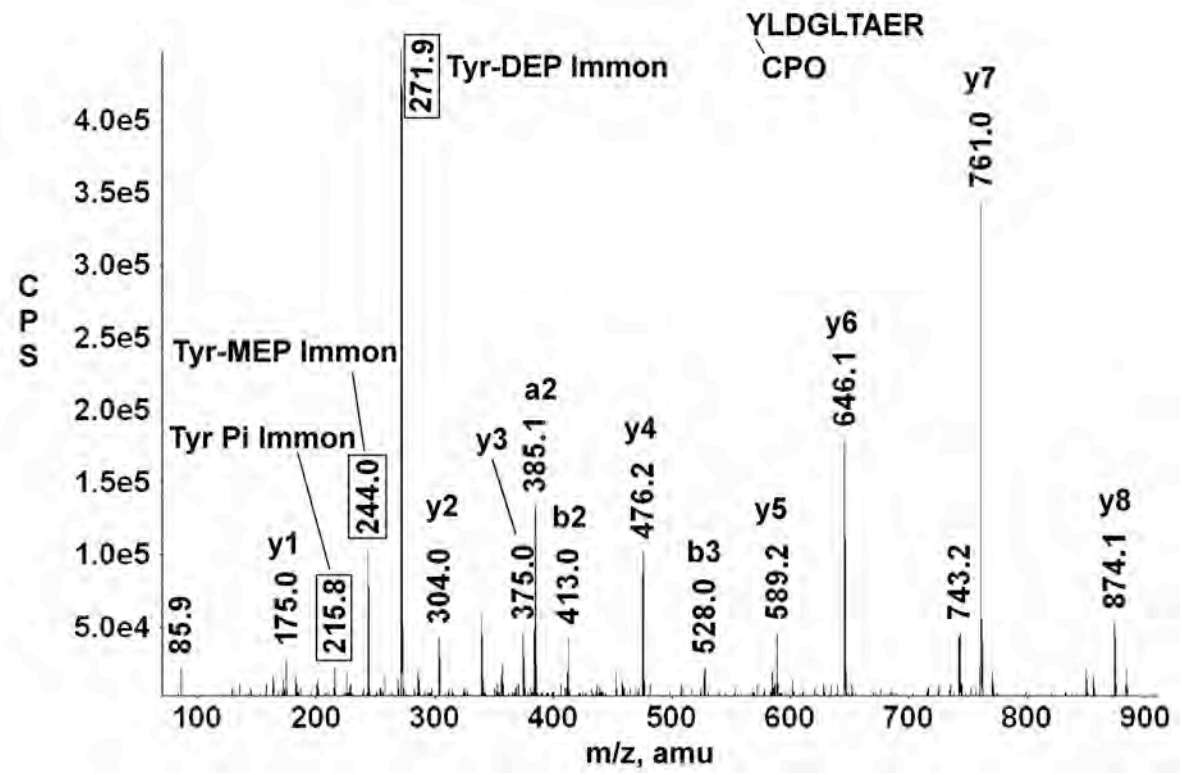


Figure 3.5. A CID mass spectrum of the CPO-labeled human keratin 2 tryptic peptide Y*LDGLTAER. The values enclosed in the boxes are the masses of the characteristic fragments for CPO-labeled tyrosine. DEP stands for diethylphosphate, MEP stands for monoethylphosphate.

Figure 3.5 illustrates the CID fragmentation of a chlorpyrifos-oxon labeled peptide. This peptide is Y*LDGLTAER from human keratin 2, which is labeled on the N-terminal residue. The parent ion is doubly-charged with an m/z of 587.3 that includes the 136 amu added mass from CPO (O,O-diethylphosphate). A y-ion series starts from 175.0 for y1 and ends at 874.1 amu for y8. Addition of 299 amu to y8, (O,O-diethylphosphate-labeled Tyr, 163 + 136 = 299 amu), yields the singly-charged parent ion mass of 1173.1 amu, which indicates the presence of the label on the N-terminus. A mass at 413.0 amu is consistent with the O,O-diethylphosphate-labeled tyrosine plus leucine, i.e. the b2-ion (164 + 136 + 113 = 413 amu), which confirms labeling of the N-terminal tyrosine. A strong signal at 385.1 amu corresponds to the labeled a2-ion. Characteristic fragments at 271.9, 244.0 and 215.8 amu are consistent with the O,O-diethylphosphate-labeled tyrosine immonium ion, the O-ethyl phosphate-labeled tyrosine immonium ion, and the tyrosine phosphate immonium ion, respectively. These are the most commonly observed characteristic ions derived from O,O-diethylphosphate-labeled, tyrosine-containing peptides and their presence fully establishes the existence of a labeled tyrosine in this peptide.

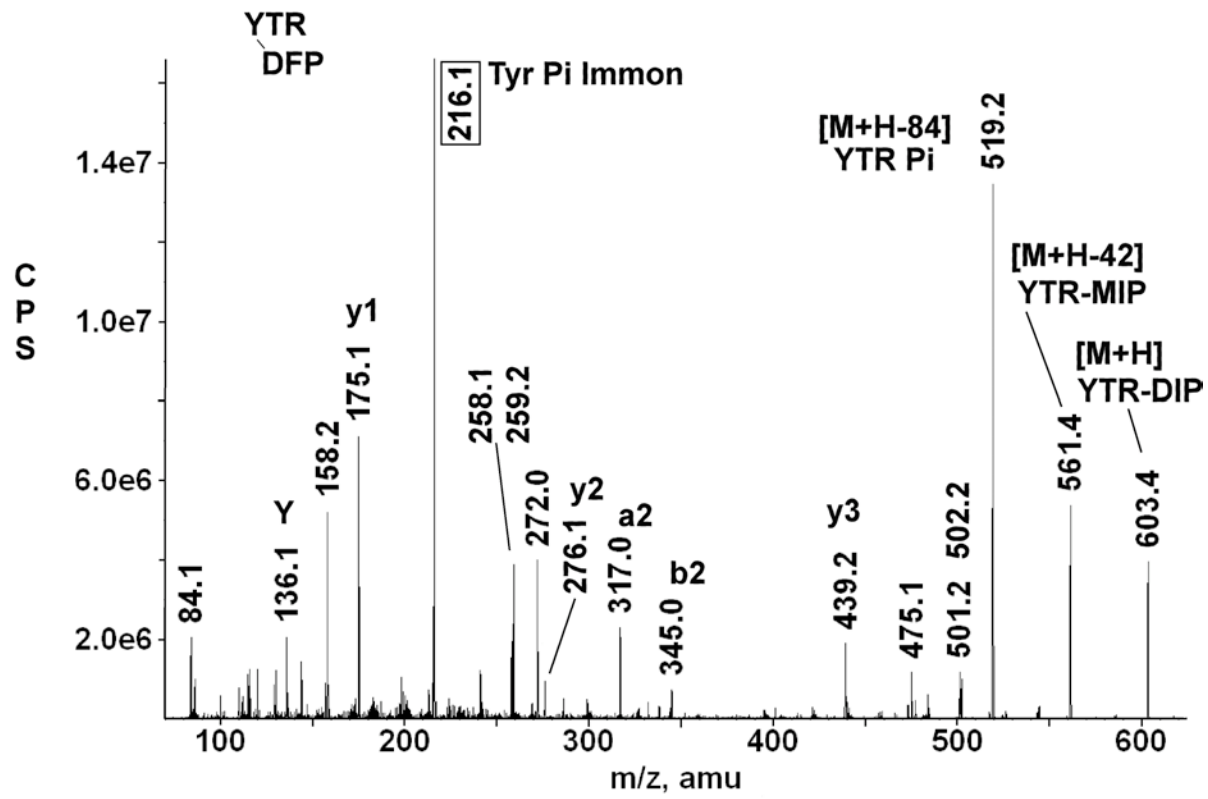


Figure 3.6. A CID mass spectrum of the DFP-labeled bovine serum albumin tryptic peptide Y*TR. The value enclosed in the box is the mass of the characteristic fragment for DFP-labeled tyrosine. MIP stands for monoisopropylphosphate, DIP stands for diisopropylphosphate.

Figure 3.6 illustrates the CID fragmentation of a diisopropylfluorophosphate-labeled peptide. This peptide is Y*TR from bovine serum albumin, which is labeled on the N-terminal residue. The parent ion is singly-charged with an m/z of 603.4 that includes the 164 amu added mass from DFP (O,O-diisopropylphosphate). Loss of isopropylene (42 amu) yields the 561.4 amu peak, and subsequent loss of a second isopropylene yields the more intense peak at 519.2 amu, which is consistent with YTR-phosphate. These neutral losses are characteristic of a diisopropylphosphate-labeled tyrosine containing peptide. The relatively large intensities of these masses illustrate the ease with which the isopropylene is eliminated from diisopropylphosphate under CID conditions. The mass difference between 519.2 and 276.1 amu (y2) is consistent with loss of Tyr-phosphate, indicating that tyrosine was the labeled residue. Loss of 80 amu from 519.2 yields the y3 peak at 439.2 amu, which further supports labeling of the tyrosine. A complete y-ion series without phosphate is present. B2 and a2 ions that retain phosphate are seen at 345.0 and 317.0 amu, respectively. A characteristic ion at 216.1 amu, consistent with Tyr-phosphate is one of the most commonly observed characteristic ions derived from O,O-diisopropylphosphate-labeled, tyrosine containing peptides. Its presence is another strong indication of the presence of an O,O-diisopropylphosphate labeled tyrosine in the peptide.

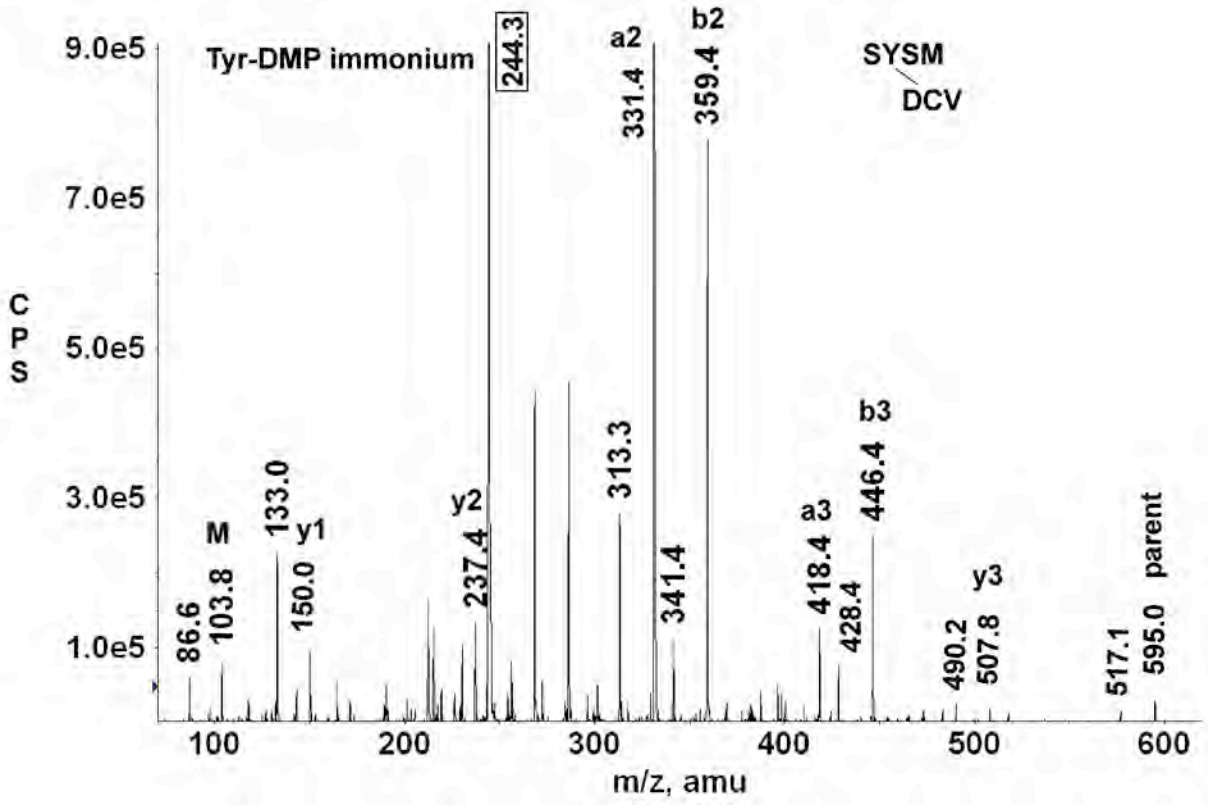


Figure 3.7. A CID mass spectrum of the DCV-labeled synthetic tryptic peptide SY*SM. The value enclosed in the box is the mass of the characteristic fragment for DCV-labeled tyrosine. DMP stands for dimethylphosphate.

Figure 3.7 illustrates the CID fragmentation of a dichlorvos-labeled peptide. This peptide is SY*SM, a synthetic peptide, which is labeled on the tyrosine. The parent ion is singly-charged with a mass of 595.0 amu that includes the 108 amu added mass from DCV (O,O-dimethylphosphate). Complete y-ion and b-ion series are present. The mass difference between y2 (237.4 amu) and y3 (507.8 amu) is 271 amu, which is consistent with the presence of O,O-dimethylphosphate labeled tyrosine (163 + 108 = 271 amu), indicating that tyrosine is the labeled residue. The mass for the b2-ion (359.4 amu) is consistent with the presence of serine (N-terminal of a b-series) plus tyrosine-O,O-dimethylphosphate (88 + 163 + 108 = 359 amu), confirming that the label is on tyrosine. The majority of the remaining masses could be attributed to loss of water, amine or CO from the fragments already described. A characteristic ion at 244.3 amu is consistent with the tyrosine-O,O-dimethylphosphate immonium ion. There is no evidence for release of methyl from the side-chain of the O,O-dimethylphosphate.

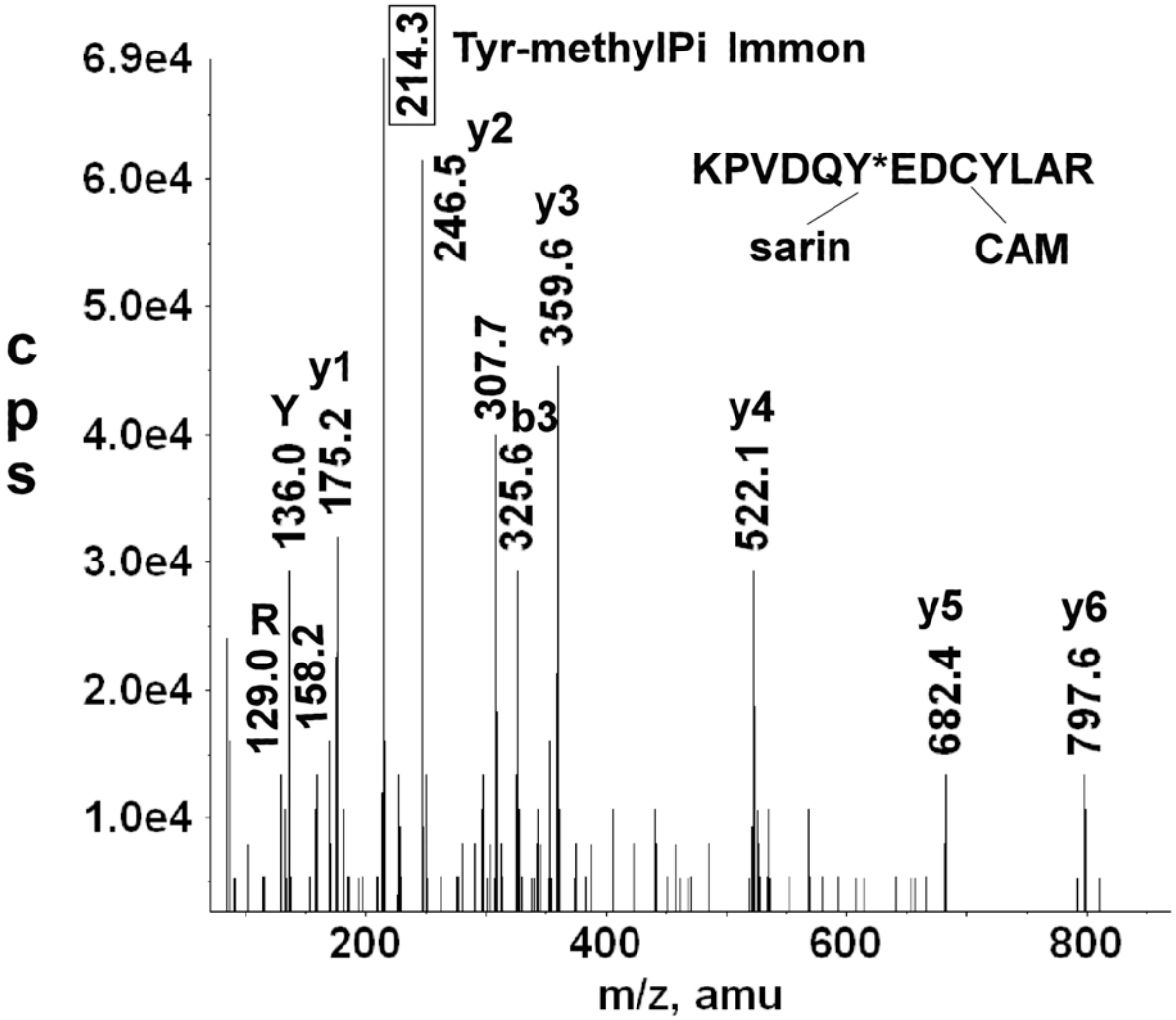


Figure 3.8. A CID mass spectrum of the sarin-labeled mouse transferrin tryptic peptide KPVDQY*EDCYLAR. The value enclosed in the box is the mass of the characteristic fragment for sarin-labeled tyrosine. MethylPi stands for methylphosphonate. CAM indicates that the cysteine is carbamidomethylated.

Figure 3.8 illustrates the CID fragmentation of a sarin-labeled peptide. This peptide is KPVDQY*EDCYLAR from mouse transferrin, which is labeled on the sixth residue from the N-terminus. The parent ion is triply-charged with an m/z of 592.0 that includes the 120 amu added mass from sarin (methylphosphonate) and a carbamidomethyl modification on cysteine (57 amu). A partial y-ion sequence from y1 to y6 includes one unlabeled tyrosine (y4); mass difference between y3 and y4 is 163 (522.1 – 359.6). The b3 ion and its dehydration product are visible at 325.6 and 307.7 amu, respectively. There is no evidence for labeling of the lysine at b1. Having thus eliminated the lysine at b1 and the tyrosine at y4, the remaining tyrosine in the sequence (y8) has been assigned as the labeled residue; no other likely candidates exist in this peptide. The mass at 214.3 amu is consistent with the tyrosine-methylphosphonate immonium ion, which is a characteristic fragment for sarin labeled tyrosine. Absence of the isopropyl group

from this characteristic fragment reaffirms the relative lability of the O-isopropyl moiety to fragmentation under CID conditions in the mass spectrometer.

OP-Reactivity of Tyrosine. Reaction of OP with tyrosine does not appear to be as specific as reaction of OP with the active site serine of the serine esterases. No consensus sequence around the labeled tyrosine was observed. Often, we were able to find multiple reactive tyrosines on a single protein. Sometimes we found more than one reactive tyrosine on a single peptide (see Table 3.1). Reactive tyrosines were typically on the surface of the protein, however not all surface tyrosines were reactive.

Of those tyrosines on a given protein that were reactive, one was generally much more reactive than the others. For example, five tyrosines from human serum albumin were found to react when plasma was treated with 200 mM FP-biotin at 37°C for 48 hours. However, only two labeled peptides, Y*TK and HPY*FYAPELLFFAK, were detected when 100 mM FP-biotin was incubated with 15 mM albumin at 22°C and pH 8 for 2 hours. Fifty-two percent of the YTK peptide was labeled while only 10% of the HPY*FYAPELLFFAK peptide was labeled. Thus Y*TK was the most reactive peptide on human serum albumin (Ding et al., 2008). Support for this assignment comes from the observation that the Y*TR peptide from bovine serum albumin was the only labeled peptide detected after reaction of equimolar amounts of protein and FP-biotin (137 mM each, reacted at 37°C and pH 8.6 for 24 hours) (Schopfer et al., 2005).

Bovine alpha tubulin provides another example of selective reaction. Out of nine peptides that were labeled by 0.5 mM CPO (pH 8.3 for 24 hours at 37°C) only one, TGTY*R, was labeled by 0.01 mM CPO under the same conditions (Grigoryan et al., 2009a). Bovine actin provides a third. Out of five peptides found on incubation with 240 mM CPO, only one, GY*SFVTTAER, gave good signals with 48 mM CPO.

Such selective reaction strongly suggests that the reactive tyrosine is somehow activated. How might such activation be manifested?

Activation of tyrosine in general. Reaction of tyrosine with OP would be expected to involve nucleophilic attack by the phenolate anion of tyrosine on the phosphorus of the OP (Benkovic and Schray, 1973). The pKa of the tyrosine phenolate is 10.1 (Ballinger and Long, 1960). Thus, at pH 8 only 1% of the typical tyrosine would be ionized. Activation could be manifested if the pKa value of selected tyrosines were lowered, thereby increasing the fraction of phenolate at pH 8.

Perturbation of pKa values can be accomplished by stabilizing or destabilizing the ionized component. A classical example is the increase in pKa for ionization of the second acidic group of a dicarboxylic acid (Kirkwood and Westheimer, 1938; Westheimer and Kirkwood, 1938). The presence of the negative charge on the first carboxylic acid causes an unfavorable electrostatic interaction for the ionization of the second, thereby increasing its pKa. Westheimer and Kirkwood calculated that a 0.8 unit difference between pKa values for carboxyls of succinic acid corresponded to a distance between carboxylates of 5 to 6 angstroms (Westheimer and Kirkwood, 1938).

By analogy with the carboxylate situation, the presence of a positive charge at a moderate distance from a developing anion could stabilize the anion and lower its pKa. Or, hydrogen-bond donors could stabilize a developing negative charge, thereby lowering the pKa.

This concept has been extended to enzymes.

Shafer and coworkers demonstrated that when the active site cysteine of papain (Cys25) is deprotonated, the pKa of His159 increases by 4.2 units (Lewis et al., 1976; Johnson et al., 1981) with a comparable decrease in the pKa of Cys 25 (Roberts et al., 1986). They attributed this change to through-space, charge-charge interactions between Cys25 and His159 (Roberts et al., 1986). They argued that charge-charge interactions do not cause the decrease in nucleophilic reactivity that is seen when pKa values are perturbed by charge-dipole interactions (Roberts et al., 1986). The consequence of this change in pKa was an increase in the fraction of nucleophilic cysteine present in the active site. The 4.2 pH unit change in pKa corresponded to a distance of 3.4 angstrom between the cysteine thiol and N3 of the histidine (Drenth et al., 1970).

This sort of pKa change is not limited to cysteines. Tyrosines with abnormally low pKa values are involved directly or indirectly in the catalytic activity of a number of enzymes including glutathione S-transferase (Atkins et al., 1993), asparaginase (Derst et al., 1994), and beta-lactamases (Lamotte-Brasseur et al., 2000). A lysine at the active center of acetoacetate decarboxylase was implicated in a 3.7 unit decrease in the pKa of a phenolic reporter group (Kokesh and Westheimer, 1971).

Activation of OP reactive tyrosines. In an effort to determine whether charge-charge interactions might be involved in the activation of the tyrosines that react with OP in our studies, we examined the x-ray structures for proteins on which OP-reactive tyrosines were found (Table 3.4). Protein structure files from the Protein Data Bank maintained by the Research Collaboratory for Structural Bioinformatics were examined using PyMOL (version 0.99rc6, DeLano, W.L. The PyMOL Molecular Graphics System, 2002, http://www.pymol.org). Forty-one tyrosines were evaluated. Two factors were considered: accessibility of the phenolic hydroxyl to solvent and the proximity of the tyrosine hydroxyl to positively charged residues. Solvent accessibility was evaluated visually using the surface feature of PyMOL. The surface that was displayed was defined by the Van der Waals surfaces of water atoms when in contact with the protein. If the phenolic hydroxyl was visible on the surface, it was considered to be solvent accessible. Positively charged groups were considered to be sufficiently close to the tyrosine hydroxyl to affect ion-pairing if they were within 6 angstroms. Choice of 6 angstrom was based on the calculations by Westheimer and Kirkwood which showed effects on the pKa of nearly 1 pK unit at this distance (Westheimer and Kirkwood, 1938).

Twenty-three of the 41 tyrosines (56%) were both solvent exposed and within 6 angstroms of a positively charged residue (Table 3.4). Ten of the instances in which the distance was greater than 6 angstroms came from bovine tubulin (alpha and beta). If tubulin is excluded from the analysis (sixteen tyrosines total), then 18 of the remaining 25 tyrosines (72%) were within ion-pairing distance of a positively charged residue. Exclusion of tubulin might be justified in light of the fact that the crystal structure was of the isolated alpha/beta dimer, whereas in solution tubulin is normally in some polymeric form (e.g., protofilaments or microtubules). Charge-charge interactions between residues on adjacent dimers might occur under those circumstances.

Of the seven non-tubulin tyrosines that were not within 6 angstroms of a positively charged residue, only two (that from pepsin and that from adenine nucleotide translocase) showed more than 10 angstroms of separation (Table 3.4). There is reason to believe that these seven residues might still enter into charge-charge interactions if the proteins were not constrained by the crystal packing. That reasoning is based on the fact that, for proteins that had more than one identical subunit in the crystal, the distances between tyrosines and their charged partners varied by up to 1 angstrom. Examples of this can be found in the measurements given

in Table 3.4 for the human serum albumin dimer, the three beta subunits in bovine ATP synthase, the three subunits of human apolipoprotein A-I, and the dimer of human kinesin 3C. This argument is consistent with the time-honored understanding that proteins in solution undergo substantial conformational change.

Though the evidence in favor of charge-charge interaction lowering the pKa of the reactive tyrosines in order to activate them is compelling, it would be naïve to suggest that other factors may not play a role. For example, several instances where a non-reactive tyrosine was found to be 3-6 angstroms from a positively-charged residue are given in Table 3.4 (see human serum albumin and human a2-glycoprotein, zinc). That these residues are not reactive can be ascribed to steric constraints. An analogous situation exists for 3-nitrotyrosine, which has a pKa of 6.5. Based on its pKa, 3-nitrotyrosine was expected to react with OP. However, nitration of human serum albumin and the peptide RYGRK with peroxynitrite resulted in a preparation that showed no reactivity toward OP (PM, unpublished observations). Absence of reactivity might again be due to steric interference, this time from the vicinal nitro group.

In summary, the above correlations strongly suggest that most, if not all, OP-reactive tyrosines are activated by the nearby presence of a positively-charged residue that is capable of forming a charge-charge ion-pair with the phenolate oxygen of the tyrosine, thereby lowering the tyrosine pKa, and enhancing its nucleophilic character for reaction with the OP.

Table 3.4. Ion-Pairing Analysis of Protein Crystal Structures; Evaluation of Interactions between OP-Labeled Tyrosines and Arginine, Lysine and/or Histidine Residues

Peptide [a]	Labeled Residue [b]	Exposed/ Buried [c]	Ion-Pair Residue [b]	Distance [d] Å
Human Serum Albumin [PDB file 1BM0, 2.50 angstrom resolution]				
There are two monomers in the unit cell for this human serum albumin structure. Measurements are given for both the alpha and beta monomers.				
Y*TK (A subunit)	Y411	Exposed	K414	5.26
			R410	4.53
Y*TK (B subunit)	Y411	Exposed	K414	5.47
			R410	6.56
HPY*FYAPELLFFAK (A subunit)	Y148	Exposed	K106	5.27
			R197	3.58
HPY*FYAPELLFFAK (B subunit)	Y148	Exposed	K106	5.65
			R197	4.10
HPYFY*APELLFFAK (A subunit)	Y150	Exposed	H242-Ne2	3.81
			R257	3.75
HPYFY*APELLFFAK (B subunit)	Y150	Exposed	H242-Ne2	3.44
			R257	4.07
MPCAEDDY*LSVVLNQLCVLHEK (A subunit)	Y452	Exposed	K436	4.47
			K432	5.69
MPCAEDDY*LSVVLNQLCVLHEK (B subunit)	Y452	Exposed	K436	4.80
			K432	6.15
QNCELFEQLGEY*K (A subunit)	Y401	Exposed	K525	4.84
QNCELFEQLGEY*K (B subunit)	Y401	Exposed	K525	3.67

Y*KAAFTECCQAADK (A subunit)	Y161	Exposed	R117	8.46
Y*LYEIAR (A subunit)	Y138	Exposed	R160	7.89
			R117	10.06

Note that Y140 was exposed to solvent and was 2.69 angstroms from a potential ion-pairing partner in R144, but this residue has not been found to be labeled by OP.

Human Transferrin

[PDB file 2HAV, 2.70 angstrom resolution]

This is the structure for apo-transferrin.

Both apo- and holo- transferrin react with OP at the same sites, and there is no effect of OP labeling on the affinity of apo-transferrin for iron.

KPVDEY*K	Y238	Exposed	K239	4.29
			H207-Ne2	4.46
KPVEEY*ANCHLAR	Y574	Exposed	H535-Ne2	4.36

Human a2-Glycoprotein, zinc

[PDB file 1T7V, 1.95 angstrom resolution]

AY*LEEECPATLR	Y161	Exposed	R73	5.03
YY*YDGKDYIEFNK	Y118	Exposed	K116	4.25
			R100	5.01
WEAEPV*VQR	Y154	Exposed	R157	5.58

Note that a number of other tyrosines were within ion-pairing distance of positively charged residues, were exposed to solvent, but were not labeled by OP: Y119 was 3.23 angstroms from H95-Nd1; Y211 was 4.97 angstroms from R183; and Y258 was 5.01 angstroms from R221.

Bovine Tubulin alpha

[PDB file 1JFF–with taxol, 3.5 angstrom resolution]

IHFPLATY*APVISAEK	Y272	Exposed	R320	4.73
EDAANNY*AR	Y103	Exposed	H107-Ne2	4.20
			H192-Nd1	5.13
GHY*TIGK	Y108	Exposed	H107-Ne2	4.67
FDLMY*AK	Y399	Exposed	R402	2.83
TGTY*R	Y83	Exposed	R229	7.47
AFVHWY*VGEGMEEGEFSEAR	Y408	Buried	H406-Ne2	11.09
FDGALNVDLTEFQTNLVPY*PR	Y262	Exposed	H266-Nd1	8.52
			R264	13.27
VGINY*QPPTVVPGGDLAK	Y357	Exposed	R373	12.75
			K370	13.04
LSVDY*GK	Y161	Exposed	R123	7.19

Bovine Tubulin beta

[PDB file 1JFF (with taxol), 3.5 angstrom resolution]

GHY*TEGAELVDSVLDVVR	Y108	Exposed	H107-Nd1	4.72
NSSY*FVEWIPNNK	Y342	Exposed	R308	4.91
EEY*PDR	Y161	Exposed	R123	6.47
Y*VPR	Y61	Exposed	H28-Nd1	10.57
			H37-Nd1	10.73
			K60	12.92

			R64	12.18
GSQQY*R	Y283	Exposed	K218	11.34
			K372	15.62
Y*LTVAAFR	Y312	Exposed	R308	11.37
			R311	11.47
INVYY*NEATGGK	Y53	Buried	H28-Ne2	6.25
Human Kinesin 3C motor domain [PDB file 3B6U, 1.80 angstrom resolution]				
There are two monomers in the unit cell for this human kinesin structure. Measurements are given for both the alpha and beta monomers.				
ASY*LEIYQEEIR (A subunit)	Y144	Exposed	R201	4.83
ASY*LEIYQEEIR (B subunit)	Y144	Exposed	R201	5.07
Bovine Actin alpha from skeletal muscle in complex with DNase I [PDB file 2A42, 1.85 angstroms resolution]				
DSY*VGDEAQSK	Y53	Exposed	K50	4.81
			K61	5.29
IWHHTFY*NELR	Y91	Exposed	H87-Ne2	5.06
DLTDY*LMK	Y188	Exposed	R256	4.32
GY*SFVTTAER	Y198	Exposed	R196	8.09
QEY*DEAGPSIVHR	Y362	Exposed	K359	6.66
			K118	9.00
SY*ELPDGQVITIGNER	Y240	Exposed	K215	6.49
Porcine Pepsin [PDB file 4PEP, 1.80 angstroms resolution]				
QYY*TVFDDR	Y310	Buried	N-Terminal	10.93
Bovine Chymotrypsinogen [PDB file 1EX3. 3.0 angstroms resolution]				
Y*TNANTPDR	Y146	Exposed	R145	5.29
Bovine ATP Synthase beta [PDB file 2CK3, 1.95 angstroms resolution]				
No crystal structure for mouse ATP synthase could be found. However, the sequence of the beta subunit of bovine ATP synthase is 98.2% identical to that of the mouse beta subunit. Therefore it is reasonable to use the bovine coordinates to represent the mouse protein.				
ATP Synthase beta is a component of mitochondrial membrane ATP synthase. The complete structure of the ATP synthase consists of two components, F(1) and F(0). F(0) is composed of 3 subunits (A, B, and C). F(1) is composed of 5 subunits (a, b, g, d, and e in a stoichiometry of 3:3:1:1:1). The three b-subunits are designated D, E and F in the crystal structure.				
ILQDY*K (D subunit)	Y381	Exposed (barely)	R412d	6.52
			R408 d	2.96
ILQDY*K (E subunit)	Y381	Exposed	R412 e	5.67

			R408 e	2.99
ILQDY*K (F subunit)	Y381	Exposed	R412f	6.56
			R408f	3.44
Mouse Adenine Nucleotide Translocase I [PDB file 2C3E, 2.80 angstroms resolution]				
No crystal structure for mouse Adenine Nucleotide Translocase I could be found. However, the sequence of bovine adenine nucleotide translocase I is 94.6% identical to that of the mouse. Therefore it is reasonable to use the bovine coordinates to represent the mouse protein.				
Y*FPTQALNFAFK	Y80	Exposed	K22	10.38
			R78	11.17
Human Apolipoprotein A-I [PDB file 2A01, 2.40 angstroms resolution]				
There are three monomers in the unit cell for this human apolipoprotein A-I structure.				
DY*VSQFEGSALGK (A subunit)	Y29	Exposed	K59	4.63
DY*VSQFEGSALGK (B subunit)	Y29	Exposed (barely)	K59	4.82
DY*VSQFEGSALGK (C subunit)	Y29	Buried	K59	4.75

a The asterisk (*) indicates the labeled tyrosine.
b Numbering is for the mature sequence.
c Exposed is defined as the phenolic oxygen of the tyrosine being visible when the surface option for the atom display is chosen in PyMOL. Buried is defined as the phenolic oxygen of the tyrosine not being visible.
d Distance is defined as the space between the phenolic oxygen of the tyrosine and its ion-pair. For arginine-tyrosine ion-pairs, the shortest distance between the phenolate oxygen and the guanidinium group is taken. For histidine-tyrosine ion-pairs, the distance between the phenolate oxygen and the nearest ring nitrogen is taken.

Comparison of OP-tyrosine adducts with OP-serine adducts. It is well accepted that activated serine residues, such as those found in the active sites of serine esterases and proteases, react with organophosphorus agents. Recently, it has become apparent that selected tyrosine residues will also react with organophosphorus agents. Not surprisingly, the physical properties of these two classes of organophosphorus adducts are distinct.

For example, serine adducts readily undergo beta elimination (McLafferty rearrangement in the mass spectrometer) to release the organophosphorus moiety, leaving behind a dehydroalanine in place of the original serine. We have found no evidence that OP-tyrosine adducts release the organophosphyl-moiety. As a consequence of the facile elimination of OP from serine adducts under CID conditions in the mass spectrometer, there are no characteristic fragments in the mass spectra of most OP-labeled serine containing peptides. The presence of the OP on the peptide is indicated by the mass of the parent ion and the presence of dehydroalanine in the fragmentation sequence. The relative stability of OP-tyrosine adducts during CID yields an abundance of characteristic fragments that are diagnostic for the nature of

the label. These fragments in combination with the parent ion mass and the characteristic steps in the masses that define the peptide sequence provide strong evidence for the presence and nature of OP-labeled tyrosines in selected peptides.

The organophosphonate FP-biotin is unique among the OP agents in that CID fragmentation in the mass spectrometer yields characteristic masses for both labeled serine and labeled tyrosine. FP-biotinylated tyrosine yields fragments at 227, 312 and 329 amu that correspond to portions of the FP-biotin label, as well as fragments at 708 and 691 amu that correspond to the FP-biotinylated tyrosine immonium ion and its deaminated derivative. FP-biotinylated serine yields the 227, 312 and 329 amu fragments, and in addition a fragment at 591 amu that corresponds to the beta eliminated form of FP-biotin.

Another useful difference in the properties of these two OP-labeled residues concerns the process of aging. Aging is a secondary reaction of OP-labeled serine that is catalyzed by active site residues in certain serine hydrolases such as cholinesterases and serine proteases. Aging results in the hydrolysis of one of the alkoxy ligands to the phosphorus, yielding the corresponding alcohol and a hydroxyl ligand on the phosphorus (Viragh et al., 1999). OP-labeled tyrosines do not undergo this secondary reaction. This difference becomes noteworthy when trying to diagnose the type of OP agent that has reacted with a sample. For example, sarin-labeled butyrylcholinesterase and soman-labeled butyrylcholinesterase both age to give the same product, methylphosphonate labeled serine (Li et al., 2007b). Thus the parent ion masses for the active site peptide from samples that were exposed to either of these agents will be the same. On the other hand, since sarin-labeled albumin and soman-labeled albumin do not age, the parent ion masses for the labeled peptides are diagnostic for the agent that was involved in the initial exposure.

Conclusion. The fragmentation patterns for OP-labeled tyrosine adducts described above should aid in the discovery of proteins and peptides labeled by OP. The presence of characteristic ion masses in the MSMS data provide assurance that a peptide is covalently modified on tyrosine by a particular OP.

These results illustrate the binding of OP to tyrosine on a variety of proteins. Tyrosine is a new target for OP binding, supplementing the traditional active site serine in serine esterases and proteases. As such, tyrosine constitutes a new binding motif for OP reactivity.

Task 4. Set up a Multiple Reaction Monitoring method to identify soman-labeled proteins, using purified proteins.

Relation to statement of work. Multiple Reaction Monitoring was used to detect pesticide exposure in a patient sample.

Carbofuran poisoning detected by mass spectrometry of butyrylcholinesterase adduct in human serum

Li H, Ricordel I, Tong L, Schopfer LM, Baud F, Mégarbane B, Maury E, Masson P, Lockridge O. Carbofuran poisoning detected by mass spectrometry of butyrylcholinesterase adduct in human serum. J Appl Toxicol. 2009 Mar;29(2):149-55. PMID: 18937214

ABSTRACT

Carbofuran is a pesticide whose acute toxicity is due to inhibition of acetylcholinesterase. Butyrylcholinesterase (BChE) in plasma is inhibited by carbofuran and serves as a biomarker of poisoning by carbofuran. The goal was to develop a method to positively identify poisoning by carbofuran. Sera from an attempted murder and an attempted suicide were analyzed for the presence of carbofuran adducts on BChE. The BChE from 1 ml of serum was rapidly purified on a 0.2 ml procainamide-Sepharose column. Speed was essential because the carbofuran-BChE adduct decarbamylates with a half-life of about 2 h. The partially purified BChE was boiled to denature the protein, thus stopping decarbamylation and making the protein vulnerable to digestion with trypsin. The labeled peptide was partially purified by HPLC before analysis by LC/MS/MS in the multiple reaction monitoring mode on the QTRAP 2000 mass spectrometer. Carbofuran was found covalently bound to Ser 198 of human BChE in serum samples from two poisoning cases. Multiple reaction monitoring triggered-MS/MS spectra positively identified the carbofuran-BChE adduct. In conclusion a mass spectrometry method to identify carbofuran poisoning in humans has been developed. The method uses 1 ml of serum and detects low level exposure associated with as little as 20% inhibition of plasma butyrylcholinesterase.

A PDF file of the published paper is attached.

Task 6. Use a second method, for example enzyme activity assays or immunoprecipitation, to confirm the identity of soman-labeled proteins from plasma.

Relation to Statement of Work. Activity assays showed that OP labeling of transferrin did not inhibit transferrin activity. The following manuscript was included in the Annual Report for the previous year. The manuscript has been revised according to the reviewers' comments and is now published. *This publication fulfills Task 6 and Task 3.*

Tyrosines of human and mouse transferrin covalently labeled by organophosphorus agents; a new motif for OP binding to proteins that have no active site serine

Li B, Schopfer LM, Grigoryan H, Thompson CM, Hinrichs SH, Masson P, Lockridge O.
Tyrosines of human and mouse transferrin covalently labeled by organophosphorus agents: a new motif for binding to proteins that have no active site serine. Toxicol Sci. 2009 Jan;107(1):144-55. Epub 2008 Oct 16. PMID: 18930948

ABSTRACT

The expectation from the literature is that organophosphorus agents (OP) bind to proteins that have an active site serine. However, transferrin, a protein with no active site serine, was covalently modified in vitro by 0.5 mM 10-fluoroethoxyphosphinyl-N-biotinamido pentyldecanamide (FP-biotin), chlorpyrifos oxon, diisopropylfluorophosphate, dichlorvos, sarin, and soman. The site of covalent attachment was identified by analyzing tryptic peptides in the mass spectrometer. Tyr 238 and Tyr 574 in human transferrin and Tyr 238, Tyr 319, Tyr 429, Tyr 491, and Tyr 518 in mouse transferrin were labeled by OP. Tyrosine in the small synthetic peptide ArgTyrThrArg made a covalent bond with diisopropylfluorophosphate, chlorpyrifos oxon, and dichlorvos at pH 8.3. These results, together with our previous demonstration that albumin and tubulin bind OP on tyrosine, lead to the conclusion that OP bind covalently to tyrosine, and that OP-binding to tyrosine is a new OP binding residue. The OP-reactive tyrosines are activated by interaction with Arg or Lys. It is suggested that many proteins in addition to those already identified may be modified by OP on tyrosine. The extent to which tyrosine modification by OP can occur in vivo and the toxicological implications of such modifications require further investigation.

A PDF file of the published paper is attached.

Task 7. Determine the limit of detection of soman-labeled proteins in human plasma.

Relation to Statement of Work. Progress on Task 7 has been made by measuring the limit of detection of soman-labeled butyrylcholinesterase in human plasma using the method of multiple reaction monitoring. This work satisfies Tasks 4 and 7.

Multiple reaction monitoring in a tandem ion trap mass spectrometer for detection of trace amounts of soman-labeled butyrylcholinesterase in human plasma

ABSTRACT

The goal was to establish a method to detect exposure to low levels of soman. Human plasma was treated ex vivo with 0.01 to 0.18 μM soman. The butyrylcholinesterase in 1 ml plasma was purified by affinity chromatography on procainamide gel followed by nondenaturing gel electrophoresis. The gel band containing butyrylcholinesterase was reduced, carbamidomethylated, and digested with trypsin. The tryptic peptides were analyzed by multiple reaction monitoring in a QTRAP 2000 mass spectrometer. Parent/daughter ion pairs 752.8/1001.5 and 752.8/1201.6 amu gave MSMS spectra that proved the peptide masses were those of butyrylcholinesterase modified by aged soman. The limit of detection was for plasma treated with 0.01 μM soman, a concentration which inhibited the butyrylcholinesterase 2%.

INTRODUCTION

The ability to detect low levels of nerve agent exposure is important for military reasons as well as for protection of civilians. Chemical nerve agents were used during the Iraq/Iran war in the 1980ies and by a religious cult in 1995 in the Tokyo subway. There is concern that they may be used again. Low level exposure is not accompanied by symptoms of toxicity. However, people can make themselves ill with worry. Methods to detect low level exposure are needed to reassure the worried well that they have not been exposed. A method that detects low dose exposure will also be useful for identifying people who illegally synthesize and handle the chemical agents.

The military laboratories have a very good method for detecting nerve agent exposure. They treat a blood sample with potassium fluoride to release the agent from its covalent binding

site on serine, and analyze the product by GC-mass spec (Adams et al., 2004; Van Der Schans et al., 2004). The drawback of this method is that it does not work with aged nerve agent. The butyrylcholinesterase soman adduct ages with a half-life of 4 min. This means that a blood sample taken 1 hour after exposure would contain no indication of exposure.

The only laboratory to date to successfully analyze sarin exposure in a small quantity of human blood from an exposed patient was the TNO Prins Maurits laboratory in the Netherlands. Fidder et al identified sarin-labeled butyrylcholinesterase peptide by LC/MS/MS (Fidder et al., 2002). Fidder purified butyrylcholinesterase from 0.5 ml plasma and digested the butyrylcholinesterase with pepsin. Our laboratory identified exposure to the pesticide carbofuran in blood from two patients (Li et al., 2009b). We purified butyrylcholinesterase from 1 ml plasma and digested with trypsin. The tryptic peptides were analyzed by multiple reaction monitoring in the mass spectrometer. The present report is the first to determine the limit of detection of soman exposure in a human blood sample.

METHODS

Human plasma treated with soman. Human plasma (1 ml) was treated with soman to final soman concentrations of 0 (control), 0.010, 0.020, 0.045, 0.090, and 0.180 μM.

Butyrylcholinesterase activity assay. Butyrylcholinesterase activity was assayed in a Gilford spectrophotometer with 1 mM butyrylthiocholine in 0.1 M potassium phosphate buffer pH 7.0 at 25˚C in the presence of 0.5 mM dithiobisnitrobenzoic acid (Ellman et al., 1961). The absorbance increase at 412 nm was recorded. The micromoles butyrylthiocholine hydrolyzed per min were calculated from the extinction coefficient of 13,600 M^{-1} cm^{-1}. Units of activity are micromoles per min.

Purification of butyrylcholinesterase from 1 ml plasma. Human plasma contains about 0.05 nmoles of butyrylcholinesterase in 1 ml. This amount was expected to be detectable in the mass spectrometer, based on studies with highly purified butyrylcholinesterase. Butyrylcholinesterase from 1 ml plasma was purified by passage over 0.2 ml of procainamide affinity gel packed in a 1.5 ml microfuge spin column. The affinity gel had been custom synthesized by Dr. Yacov Ashani (Grunwald et al., 1997) and had a specific activity of 34 μmoles procainamide bound per ml Sepharose. The gel was equilibrated with 2 ml of 20 mM potassium phosphate pH 7.0 buffer. One ml of plasma was loaded on the column by gravity flow at a rate of 1 ml in 10 min. Centrifugation was not used for this step because binding was more complete when loading was slow. The column was washed 4 times with 1 ml of 0.2 M NaCl in 20 mM potassium phosphate pH 7.0 buffer. The column was eluted with 0.5 ml of 1 M sodium chloride in 20 mM potassium phosphate pH 7.0 buffer to remove the butyrylcholinesterase. Soman-inhibited as well as uninhibited butyrylcholinesterase bound to the affinity gel.

The partially purified butyrylcholinesterase was concentrated and desalted in a microcon ultracentrifuge device YM3, MW cutoff 3K. The final volume was 50 μl.

The butyrylcholinesterase was further purified by gel electrophoresis on a nondenaturing 4-30% polyacrylamide gradient gel. A nondenaturing gel rather than an SDS gel was used because butyrylcholinesterase separates well from albumin only on a nondenaturing gel. The gel

was stained for butyrylcholinesterase activity with butyrylthiocholine iodide as substrate (Karnovsky and Roots, 1964). Bands corresponding to butyrylcholinesterase tetramers were cut out and processed for mass spectrometry.

In-gel digestion with trypsin. Protein in the gel slices was reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin as described (Peeples et al., 2005). Peptides were extracted with 60% acetonitrile, 0.1% trifluoroacetic acid, dried in a vacuum centrifuged and dissolved in 80 µl of 5% acetonitrile, 0.1% formic acid. The peptide solution was subjected to liquid chromatography tandem mass spectrometry (LC/MS/MS) using the multiple reaction monitoring feature of the Applied Biosystems QTRAP mass spectrometer.

LC/MS/MS. The QTRAP 2000 tandem ion trap mass spectrometer (Applied Biosystems, Foster City, CA) was set up for multiple reaction monitoring. The masses of the parent and transition ions were selected based on our experience with purified human butyrylcholinesterase. We used the quadruply charged parent ion for unlabeled butyrylcholinesterase as well as for aged soman butyrylcholinesterase. The transition ions were the singly charged y9 and y11 daughter ions. A reference butyrylcholinesterase peptide was also monitored. The selected reference peptide coeluted with the butyrylcholinesterase active site peptide. The reference peptide reassured that the method was working. Table 7.1 lists the parameters we used for multiple reaction monitoring.

Table 7.1 Multiple reaction monitoring parameters for detection of aged soman-labeled butyrylcholinesterase tryptic peptide.

sequence	status	charge of parent ion	parent ion m/z	daughter ion m/z	
SVTLFGESAGAASVSLHLLSPGSHSLFTR	unlabeled	4	733.8	1001.5	y9
SVTLFGESAGAASVSLHLLSPGSHSLFTR	unlabeled	4	733.8	1201.6	y11
SVTLFGESAGAASVSLHLLSPGSHSLFTR	aged soman	4	752.8	1001.5	y9
SVTLFGESAGAASVSLHLLSPGSHSLFTR	aged soman	4	752.8	1201.6	y11
AILQSGSFNAPWAVTSLYEAR	reference	3	761.5	839.4	y7
AILQSGSFNAPWAVTSLYEAR	reference	3	761.5	1009.5	y9

RESULTS

Inhibition of butyrylcholinesterase activity. The soman treated plasma samples were tested for butyrylcholinesterase activity to determine the percent inhibition. Table 7.2 shows that the lowest concentration of soman inhibited butyrylcholinesterase 2% and the highest inhibited butyrylcholinesterase nearly 100%.

Table 7.2. Butyrylcholinesterase (BChE) inhibition by soman.

soman, μM	BChE activity, u/ml	% inhibition	molar ratio soman:BChE	expected % inhibition
0	3.20	0	0:52	0
0.010	3.13	2	10:52	19
0.020	2.32	27	20:52	38
0.045	1.05	53	45:52	86
0.090	0.03	99.1	90:52	100
0.180	0.02	99.4	180:52	100

An activity of 3.20 units/ml calculates to a butyrylcholinesterase concentration of 0.052 μM. This value was calculated from the molecular weight of 85,000 for BChE monomer, and the specific activity of 720 units/mg for pure butyrylcholinesterase.

The percent of butyrylcholinesterase inhibited was less than expected from a comparison of the molar concentration of racemic soman and of butyrylcholinesterase. The 0.010 μM soman inhibited only 2% of the butyrylcholinesterase activity, but had been expected to inhibit 19%. The 0.045 μM soman inhibited 53% of the activity but had been expected to inhibit 86%. If one assumes the soman concentrations are correct, then the lower than expected inhibition indicates that some of the stereoisomers of soman are not reacting with butyrylcholinesterase.

Multiple reaction monitoring to detect soman adduct of butyrylcholinesterase. Soman ages with a half-life of about 4 min when it is bound to butyrylcholinesterase. Therefore only aged soman adducts were expected. The multiple reaction monitoring parameters were designed to detect the aged soman adduct. Figure 7.1 shows the elution times for the parent/daughter ion masses stipulated in Table 7.1. The aged soman butyrylcholinesterase peptide elutes at 43.64 min.

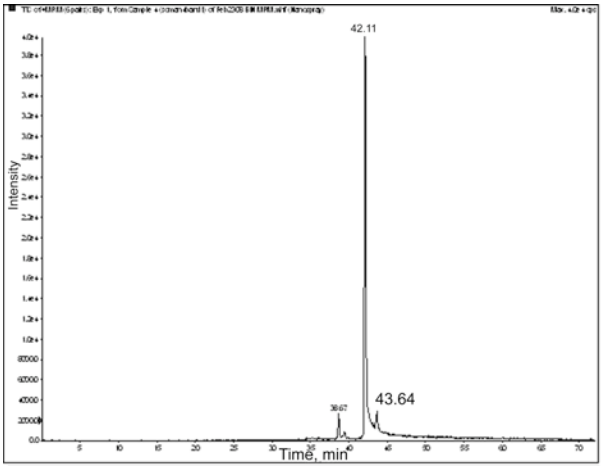


Figure 7.1. Ion chromatogram of butyrylcholinesterase peptides. The multiple reaction monitoring program was set up to recognize only peptides with the parent/daughter ion masses listed in Table 7.1. The intense peak at 42.11 min is the reference peptide AILQSGSFNAPWAVTSLYEAR. The peak at 43.64 min is the active site peptide SVTLFGESAGAASVSLHLLSPGSHSLFTR labeled with aged soman.

Figures 7.2, 7.3, and 7.4 show the parent/daughter ion pairs detected by the mass spectrometer in panel A, and the MRM-triggered MSMS spectrum for the quadruply charged parent ion 752.8 m/z in panel B.

In Figure 7.2A the plasma treated with 0.090 μM soman (99% inhibition of butyrylcholinesterase) has 752.8/1001.5 and 752.8/1201.6 amu parent/daughter ions. Fragmentation of parent ion 752.8 in Figure 7.2B yields masses that confirm the sequence SVTLFGESAGAASVSLHLLSPGSHSLFTR of human butyrylcholinesterase. Evidence that the peptide is labeled with aged soman comes from the mass of the parent ion. There are no dehydroalanine ions in Figure 7.2B.

In Figure 7.3A the plasma treated with 0.020 μM soman (27% inhibition of butyrylcholinesterase) has 752.8/1001.5 and 752.8/1201.6 amu parent/daughter ions. Fragmentation of parent ion 752.8 in Figure 7.3B yields masses that fit the sequence SVTLFGESAGAASVSLHLLSPGSHSLFTR of human butyrylcholinesterase. The MSMS spectrum in Figure 7.3B includes three dehydroalanine ions, marked with the symbol Δ. The dehydroalanine ions are characteristic of OP-labeled serine. The y24, y25, and a27 dehydroalanine ions are consistent with the interpretation that the aged soman label is on serine 8 in the sequence SVTLFGE**S**AGAASVSLHLLSPGSHSLFTR.

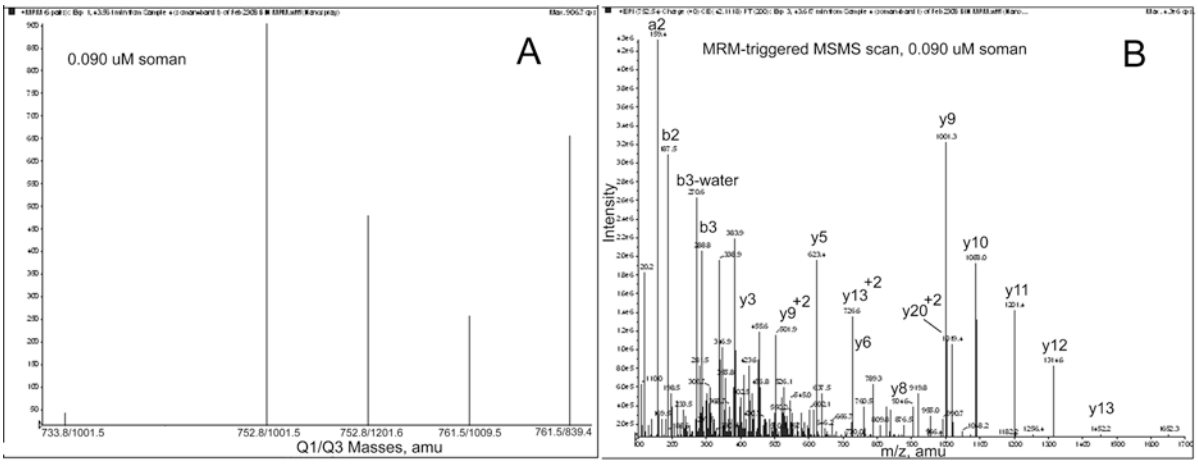


Figure 7.2. Multiple reaction monitoring (MRM) detects aged soman labeled butyrylcholinesterase peptide in plasma treated with 0.090 μM soman (99% inhibited). Panel A) Parent/daughter ions for the aged soman butyrylcholinesterase peptide are present at 752.8/1001.5 and at 752.8/1201.6 amu. B) MRM-triggered MSMS scan for the aged soman butyrylcholinesterase tryptic peptide. The masses of these ions prove that the parent ion is the aged soman-labeled peptide SVTLFGESAGAASVSLHLLSPGSHSLFTR.

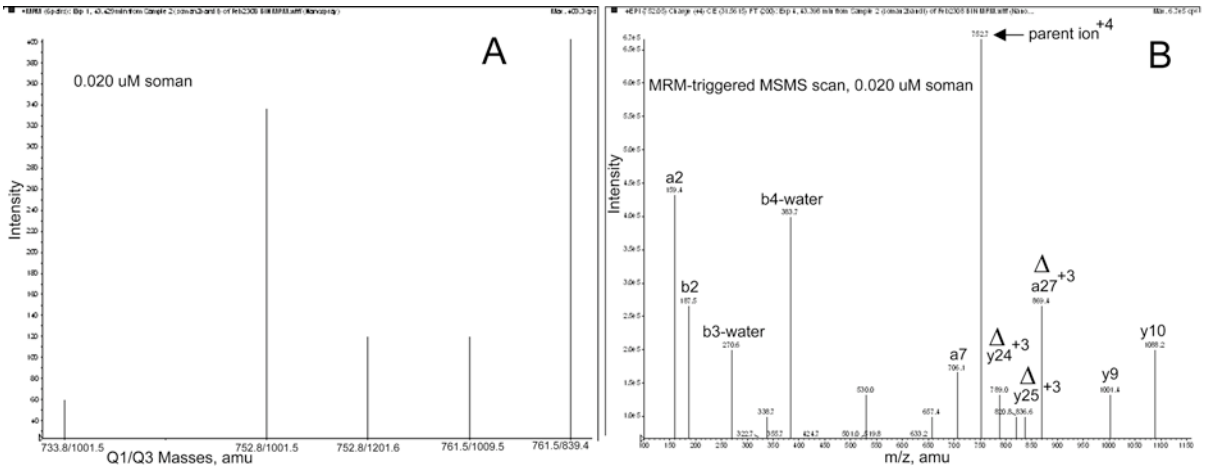


Figure 7.3. Multiple reaction monitoring detects aged soman labeled butyrylcholinesterase peptide in plasma treated with 0.020 µM soman (27% inhibited). Panel A) Parent/daughter ions for the aged soman butyrylcholinesterase peptide are present at 752.8/1001.5 and at 752.8/1201.6 amu. B) MRM-triggered MSMS scan for the aged soman butyrylcholinesterase tryptic peptide. The masses of these ions prove that the parent ion is the aged soman-labeled peptide SVTLFGESAGAASVSLHLLSPGSHSLFTR. Ions marked with Δ contain dehydroalanine in place of the active site serine and aged soman.

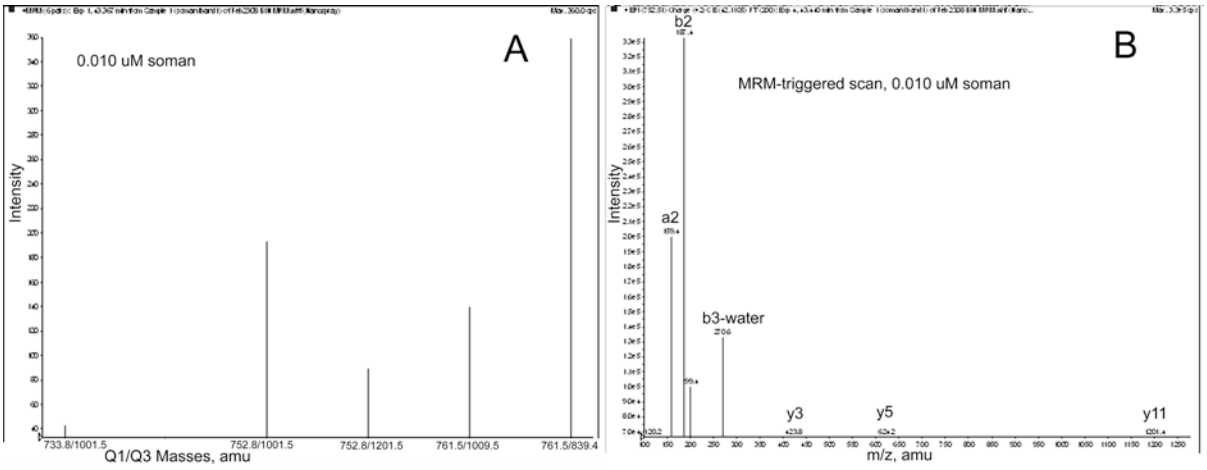


Figure 7.4. Multiple reaction monitoring detects aged soman labeled butyrylcholinesterase peptide in plasma treated with 0.010 µM soman (2% inhibited). Panel A) Parent/daughter ions for the aged soman butyrylcholinesterase peptide are present at 752.8/1001.5 and at 752.8/1201.6 amu. B) MRM-triggered MSMS scan for the aged soman butyrylcholinesterase tryptic peptide.

In Figure 7.4A the plasma treated with 0.010 µM soman (2% inhibition of butyrylcholinesterase) has 752.8/1001.5 and 752.8/1201.6 amu parent/daughter ions. Fragmentation of parent ion 752.8 in Figure 7.4B yields masses that fit the sequence SVTLFGESAGAASVSLHLLSPGSHSLFTR of human butyrylcholinesterase. The number of

ions is small and the intensity of y3, y5, and y11 ions is weak. However, the masses are correct for the butyrylcholinesterase peptide.

Control plasma samples that had not been treated with soman had no 752.8/1001.5 and 752.8/1201.6 amu parent/daughter ions.

It is concluded that the multiple reaction monitoring method is capable of detecting a soman exposure that inhibits as little as 2% of the butyrylcholinesterase enzyme. Furthermore, exposure can be detected by analyzing as little as 1 ml of plasma.

DISCUSSION

The multiple reaction monitoring method we have developed for analyzing soman exposure is very sensitive and requires only 1 ml of human plasma. The major difficulty in the method is the purification of butyrylcholinesterase from the small volume of 1 ml. It is essential to achieve a high degree of purity before subjecting the tryptic peptides to LC/MS/MS. If the peptide mixture is too complex, contaminating ions suppress ionization of the butyrylcholinesterase peptide and no butyrylcholinesterase ions are detected.

In this report we used the QTRAP 2000 mass spectrometer. A more sensitive mass spectrometer, for example the QTRAP 4000, may detect even lower levels of exposure.

Conclusion: A method has been developed that detects soman exposure at levels so low that only 2% of the plasma butyrylcholinesterase is inhibited. The method uses multiple reaction monitoring in a tandem ion trap mass spectrometer. The method does not distinguish between exposure to soman, sarin, cyclosarin, and VX because adducts of these nerve agents dealkylate to yield the same mass.

KEY RESEARCH ACCOMPLISHMENTS

- Additional evidence has been developed to support the conclusion that OP make a covalent bond with tyrosine. This strengthens our finding of a new motif for OP binding to proteins that have no active site serine.
- Mass spectrometry has identified covalent binding of OP to tyrosine in 12 proteins. This finding is contrary to the expectation that OP bind exclusively to serine.
- A multiple reaction monitoring (MRM) mass spectrometry method proved that two patients had been poisoned with the pesticide carbofuran.
- A mass spectrometry method was developed to determine the limit of detection of soman exposure in a human.

REPORTABLE OUTCOMES

- Published papers (pdf copy attached)

Li B, Nachon F, Froment MT, Verdier L, Debouzy JC, Brasme B, Gillon E, Schopfer LM, Lockridge O, Masson P. Binding and hydrolysis of soman by human serum albumin.
Chem Res Toxicol. 2008 Feb;21(2):421-31. Epub 2007 Dec 29. PMID: 18163544

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Lockridge O, Xue W, Gaydess A, Grigoryan H, Ding SJ, Schopfer LM, Hinrichs SH, Masson P. Pseudo-esterase activity of human albumin: slow turnover on tyrosine 411 and stable acetylation of 82 residues including 59 lysines. J Biol Chem. 2008 Aug 15;283(33):22582-90. Epub 2008 Jun 24.PMID: 18577514

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- In press paper (pdf copy attached)

Grigoryan H, Li B, Anderson EK, Xue W, Nachon F, Lockridge O, Schopfer LM. Covalent binding of the organophosphorus agent FP-biotin to tyrosine in eight proteins that have no active site serine. Chem Biol Interact 2009 in press

CONCLUSION

Our mass spectrometry results contradict the dogma that serine esterases and serine proteases are the only class of proteins modified by exposure to OP. We have identified 12 proteins that are labeled by OP. The significance of this finding is in diagnosis of OP exposure. It now becomes possible to look for several proteins in human tissues, in addition to butyrylcholinesterase and acetylcholinesterase, for evidence of OP exposure. In vivo exposure to the pesticide carbofuran was identified using the multiple reaction monitoring function of the QTRAP mass spectrometer. The limit of detection of exposure to soman was determined to be a dose that inhibited plasma butyrylcholinesterase 2%.

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Binding and Hydrolysis of Soman by Human Serum Albumin

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Human plasma and fatty acid free human albumin were incubated with soman at pH 8.0 and 25 °C. Four methods were used to monitor the reaction of albumin with soman: progressive inhibition of the aryl acylamidase activity of albumin, the release of fluoride ion from soman, ^{31}P NMR, and mass spectrometry. Inhibition (phosphonylation) was slow with a bimolecular rate constant of 15 ± 3 M^{-1} min^{-1}. MALDI-TOF and tandem mass spectrometry of the soman−albumin adduct showed that albumin was phosphonylated on tyrosine 411. No secondary dealkylation of the adduct (aging) occurred. Covalent docking simulations and ^{31}P NMR experiments showed that albumin has no enantiomeric preference for the four stereoisomers of soman. Spontaneous reactivation at pH 8.0 and 25 °C, measured as regaining of aryl acylamidase activity and decrease of covalent adduct (pinacolyl methylphosphonylated albumin) by NMR, occurred at a rate of 0.0044 h^{-1}, indicating that the adduct is quite stable ($t_{1/2} = 6.5$ days). At pH 7.4 and 22 °C, the covalent soman−albumin adduct, measured by MALDI-TOF mass spectrometry, was more stable ($t_{1/2} = 20$ days). Though the concentration of albumin in plasma is very high (about 0.6 mM), its reactivity with soman (phosphonylation and phosphotriesterase activity) is too slow to play a major role in detoxification of the highly toxic organophosphorus compound soman. Increasing the bimolecular rate constant of albumin for organophosphates is a protein engineering challenge that could lead to a new class of bioscavengers to be used against poisoning by nerve agents. Soman-albumin adducts detected by mass spectrometry could be useful for the diagnosis of soman exposure.

1. Introduction

Albumin is an abundant protein that represents 50–60% of the total protein in human plasma and body fluids. Its concentration in plasma is about 0.6 mM. Albumin displays both an esterase activity (*1*) and an aryl acylamidase activity (*2*, *3*). The topology of the esterase/amidase active site of albumin has been probed by site-directed mutagenesis (*4*) and X-ray structure determination of several drug−albumin complexes (*5*). Tyr411 was determined to be the catalytic nucleophile in these reactions.

Albumin is also known to bind organophosphates (OPs[1]) and carbamates (*6–8*) and to react with them (*9–11*). OPs and certain carbamates are actually hydrolyzed by albumin through transient phosphylation/alkylation of its active site (*12–17*). The residue that reacts with OPs was proven to be a tyrosine (*9*−*11, 14, 18*). DF^{32}P-labeling of human albumin followed by peptide sequencing showed that the labeled tyrosine is in the tetrapeptide sequence Arg-*Tyr*-Thr-Lys (*9*). Arg and Tyr were subsequently identified as Arg 410 and Tyr 411, the key residues of the esteratic site of HSA (*4*). Recently, DFP-inhibition of the aryl acylamidase (AAA) activity of FAF-HSA confirmed that Tyr 411 is also the nucleophilic pole of the albumin AAA activity (*2*). Finally, MALDI-TOF mass spectrometry provided direct evidence that the OPs chlorpyrifos-oxon, dichlorvos, DFP, and sarin bind covalently to human albumin at Tyr 411 (*19*).

In the present work, we investigated the reaction of human albumin with soman. MALDI-TOF and quadrupole tandem MS/MS mass spectrometry of the soman−albumin adduct showed that Tyr411 was phosphonylated. Unlike soman adducts of butyrylcholinesterase, the albumin−soman adduct did not age, that is, lose its pinacolyl chain. Covalent docking simulations and ^{31}P NMR experiments indicated that there was no enantiomeric preference of albumin for the stereoisomers of soman. Kinetic parameters of albumin phosphonylation by soman and subsequent dephosphonylation were determined.

Our results could have application for the detection of soman exposure in humans. Mass spectrometry could be used to detect soman−albumin adducts. In addition, antibodies to the soman−albumin adduct could be generated for use in a rapid antibody-based assay of soman exposure. Lastly, mutants of albumin could lead to a new class of scavengers against OP poisoning.

2. Experimental Procedures

2.1. Chemicals. Fatty acid-free human albumin (FAF-HSA) and porcine pepsin were from Sigma Chemical Co. (Saint Quentin

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[1] Abbreviations: AAA, aryl acylamidase; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; DFP, diisopropylfluorophosphate; FAF-HSA, fatty acid-free human serum albumin; HSA, human serum albumin; MP, methylphosphonate; PMP, pinacolyl methylphosphonate; o-NTFNAC, *N*-(*o*-nitrophenyl)trifluoroacetamide; OP, organophosphorus ester.

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Fallavier, France). o-NTFNAC was a gift from Dr. Sultan Darvesh (Dalhousie University, Halifax, Canada). Racemic soman (pinacolyl methylfluorophosphate) dissolved in isopropanol was from CEB (Vert-le-Petit, France). All other chemicals were of biochemical grade.

The concentration of racemic soman in the stock solution was determined by programmed temperature gas chromatography after hydrolysis into methylpinacolyl phosphonic acid and derivatization with pentafluorobenzyl bromide (*20*). It was 4.73 mg/mL (26 mM). ^{31}P NMR spectra of 2 mM soman in deuterated dimethylsulfoxide were recorded at 27 °C on a Bruker spectrometer (AM400, 9.4 T). The spectra showed that the four stereoisomers are present in nearly equimolar amounts. Soman has two chiral centers. The absolute configuration of the four diastereoisomers is known. P_S, P_R, C_S, and C_R correspond to the old notation P− P+, C+, and C−, respectively. Thus, isomers P_SC_S = P−C+, P_SC_R = P−C−, P_RC_R = P+C−, and P_RC_S = P+C+. Stereoisomers P_SC_S and P_SC_R are the most active toward the biological target acetylcholinesterase (*21*).

2.2. Aryl Acylamidase Enzymatic Assay of Albumin. The AAA activity of albumin was assayed with o-NTFNAC (2 mM) as the substrate in 60 mM Tris/HCl buffer at pH 8.0 at 25 °C. A 60 mM stock solution of o-NTFNAC was prepared in 50% water/acetonitrile (v/v). Because isopropanol is the solvent for soman, isopropanol was also added to the buffer in the control assay for the AAA activity of albumin in the absence of soman. The final concentration of acetonitrile in the assay was 3.3% and that of isopropanol was 2%. The release of the phenolic product (*o*-nitroaniline) was monitored for 5 min at 430 nm (ε = 3954 M^{-1} cm^{-1}) according to Darvesh et al. (*22*). Because of the low enzymatic activity of albumin with o-NTFNAC (*3*), high concentrations of FAF-HSA were used in assays (0.075 mM final). Measured rates were corrected for spontaneous hydrolysis of o-NTFNAC.

2.3. Mass Spectrometry (MALDI-TOF and Quadrupole MS/MS) of the Soman−Albumin Adduct from Human Plasma. One hundred microliters of human plasma was mixed with 2.3 μL of stock soman (26 mM in isopropanol) to give a reaction mixture containing 600 μM soman (in 2.3% isopropanol). The mixture was incubated at room temperature for 3–7 days before processing. No buffer was added to the plasma during this step. The pH of a 10 μL aliquot was reduced to pH 2.3 by adding 10 μL of 1% trifluoroacetic acid. Proteins were digested with 0.5 μg of pepsin for 2 h at 37 °C. At pH 2.3, selective proteolysis at the C-terminal side of the leucine and phenylalanine residues is expected. There was no need to denature the proteins or to reduce and alkylate the disulfide bonds because the peptides of interest were released without these added steps. Peptides were separated on a C18 reverse phase column on a Waters 625 LC system with a 40 min gradient starting with 85% buffer A (0.1% trifluoroacetic acid in water), 15% buffer B (acetonitrile containing 0.07% trifluoroacetic acid), and ending with 65% buffer A and 35% buffer B. One milliliter fractions were reduced in volume to 200 μL in a vacuum centrifuge, and 1 μL was analyzed by MALDI-TOF with a 2,5-dihydroxybenzoic acid matrix. Mass spectra were acquired with the Applied Biosystems Voyager DE-PRO MALDI-TOF mass spectrometer in linear positive ion mode. The spectrometer was calibrated using standard peptides (Applied Biosystems Mixture 1). A control plasma sample was identically treated, except that it was incubated with 3.1% isopropanol rather than with soman.

MS/MS spectra were acquired on a Q-Trap 2000 triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA) with a nano electrospray ionization source. Samples were infused into the mass spectrometer at 0.35 μL/min via a fused silica emitter (360 μm o.d., 20 μm i.d., 15 μm taper, New Objective, Woburn, MA) using a Harvard syringe pump to drive a 25 μL Hamilton syringe equipped with an inline 0.25 μm filter. Samples were sprayed with 50% acetonitrile and 0.1% formic acid. Mass spectra were calibrated using fragment ions generated from collision-induced dissociation of Glu fibrinopeptide B (Sigma). Enhanced product ion scans were obtained with a collision energy of 50 ± 5 V and a pure nitrogen gas pressure of 4 × 10^{-5} Torr. The final enhanced product ion scan was the average of 105 scans. Ions were identified by manual sequencing.

2.4. ^{31}P NMR Spectroscopy. ^{31}P NMR spectra for the reaction between albumin (0.78 mM or 1.3 mM) and soman (1.3 mM) in 60 mM Tris/HCl buffer at pH 8.0 were recorded at 27 °C on a Bruker AM400NB spectrometer operating at 162 MHz. The spectra were acquired using successive 8 acquisition blocks of 5,000 accumulated scans (acquisition time: 3 h/spectrum) using 30 kHz spectral width, 32,000 acquisition points, and a composite pulse proton decoupling (CPD mode). The external reference for chemical shifts was 85% (w/v) H_3PO_4. Spectra were compared to the spectrum of soman in buffer and the spectrum of methyl pinacolyl phosphonate (MPP) in the presence and absence of albumin. MPP was made by the complete hydrolysis of soman in 5 N sodium hydroxide.

2.5. Kinetic Studies of the Reaction of Albumin with Soman. 2.5.1. Residual AAA Activity. FAF-HSA (780 μM) was incubated with 120 to 1300 μM concentrations of racemic soman in 60 mM Tris/HCl buffer at pH 8.0 at 25 °C. The time dependence of the inhibition of albumin by soman was monitored by following the residual AAA activity of albumin. Measurements of the AAA residual activity were performed on 100 μL aliquots of reaction mixture, using the sampling method (*23*).

2.5.2. Monitoring the Release of Fluoride from Soman. The release of fluoride, that is, the leaving group of soman, upon the reaction of soman with albumin and spontaneous hydrolysis was monitored by ionometry using a thermostatted ionometer (Radiometer IONcheck 45) equipped with an ion-selective electrode for fluoride (ISE 301F). FAF-HSA (780 μM) was incubated with 520, 780, and 1300 μM concentrations of racemic soman in 60 mM Tris/HCl buffer at pH 8.0 at 25 °C, and the concentration of released fluoride was assayed. The spontaneous hydrolysis of soman was determined under the same conditions.

2.5.3. Kinetic ^{31}P NMR Spectroscopy. To follow the phosphonylation reaction, 1.33 mM FAF-HSA was incubated with 1.35 mM soman in 60 mM Tris/HCl buffer at pH 8.0 at 25 °C in the presence of deuterated DMSO (10% v/v final). The successive spectra were recorded every 20 min (800 scans) over 4 h. Concentrations of the different species were plotted as a function of time. To follow the reactivation of phosphonylated albumin, 1.3 mM FAF-HSA was reacted with one equivalent of racemic soman in 60 mM Tris/HCl buffer at pH 8.0. Then, the phosphonylated albumin solution was maintained at 25 °C, and spectra were recorded every 2 h over 60 h. Kinetic constants for the spontaneous hydrolysis of soman, phosphonylation of albumin, and reactivation of albumin (dephosphonylation) were determined by fitting data against the numeric solution of differential equations that describe Scheme 1, using Mathematica 5 (Wolfram Research).

2.5.4. Kinetic Mass Spectrometry. The stability of the covalent soman−albumin adduct was measured on a MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems). Human albumin was labeled by incubating a 1 mg/mL solution (15 μM) in 10 mM Tris/HCl at pH 8.0 with 200 μM soman for 24 h at room temperature. After this time, the concentration of active soman was negligible as determined by measuring the inhibition of human butyrylcholinesterase. The albumin solution was diluted to 0.1 mg/mL with 0.01% sodium azide in water and the pH adjusted to 7.4. The 200 μL solution was stored at 22 °C. Every 2 or 3 days, a 10 μL aliquot was acidified with 10 μL of 1% trifluoroacetic acid and digested with 2 μL of 1 mg/mL porcine pepsin (dissolved in 10 mM HCl). The digest was incubated at 37 °C for 1–4 h, and then 0.5 μL was spotted on a MALDI target plate. The dry spot was overlaid with 0.5 μL of 10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid. MS scans in reflector mode were acquired at 3000 V by summing 500 laser shots per scan. The percent label on Tyr 411 was calculated from cluster areas. Peptide masses acquired in reflector mode are monoisotopic, which makes their mass about 1 amu lower than the average mass acquired in linear mode.

2.6. Molecular Modeling. 2.6.1. Noncovalent Docking. Docking calculations were carried out using two programs: (1) Autodock, version 3.0.5, with the Lamarckian genetic algorithm (LGA (*24*)) and (2) Gold, version 3.1 (CCDC Software, Ltd). Docking with Autodock employed the following procedure. The molecular models of the four diastereoisomers of soman were built and minimized with the MM2 force field of Chem3D (Cambridge soft.). The structure of HSA was prepared from the crystal structure of its complex with indoxyl sulfate (pdb code 2bxh) or warfarin (pdb code 2bxd) to take into account the heterogeneity of conformation of Val433. Molecules of water and ligands were removed from the model. Soman and HSA were further prepared using Autodock Tools 1.4 (*25*). The 3D affinity grid box was designed to include the full pocket near Tyr411. The number of grid points in the *x*-, *y*-, and *z*-axes was 60, 60, and 60 with grid points separated by 0.375 Å. Docking calculations were set to 100 runs. At the end of the calculation, Autodock performed cluster analysis. Docking solutions with ligand all-atom root-mean-square deviation (rmsd) within 1.0 Å of each other were clustered together and ranked by the lowest energy representative. The lowest-energy solution was accepted as the one most representative of the soman−HSA complex. Docking with Gold employed the following procedure. Protein structures were protonated and minimized by conjugate gradients using Gromacs 3.3 (*26*, *27*) and the parameter set 53A6 (*28*). Ligands were built into the Sybyl 7.2 package (Tripos Inc.). The Mopac-PM3 (*29*) semiempirical method was chosen for geometry optimization and calculation of atomic charges. The cavity was defined as residues having atoms up to 15 Å from the Tyr411 hydroxyl group. We used long search settings for the Gold GA: 40 docking with parameters corresponding to the default 200% search efficiency settings. The final selection of conformers was based on the two scoring functions, Goldscore (*30*) and Chemscore (*31*).

2.6.2. Covalent Docking. This simulation was carried out using Gold, version 3.1 with GA settings similar to those for noncovalent docking. We used the same proteins as before, and the results were scored using both Goldscore and Chemscore functions. The fluorine atom from each of the four soman stereoisomers was replaced by a hydroxyl group, and the phosphorus stereochemistry was inverted to get the right configuration when the phosphorus atom binds to the Tyr411 Oγ. The highest score isomers were chosen as the ones giving the lowest ΔH_f when estimating the energy of the system with Mopac-PM3. To reduce the number of atoms in our system, we removed residues having all atoms further than 5 Å from any soman atoms.

3. Results

3.1. Mass Spectrometry to Identify the Covalent Binding of Soman to Tyrosine 411. Peptic digests of soman-labeled and control human plasma were separated by HPLC. Matched fractions from the two preparations were examined by MALDI-TOF mass spectrometry. MALDI-TOF signals for soman−albumin and control albumin peptides are shown in Figure 1. A peptide with mass 1880 amu is present in fraction 21 from the soman-labeled preparation (Figure 1, panel B). This mass is consistent with the peptide (L)VRY*TKKVPQVSTPTL(V) (1718 amu, from residues 409–423 of the mature albumin sequence) with an added mass of 162 amu from soman. A peptide with mass 1993 amu is present in fraction 23 from the soman-labeled preparation (Figure 1, panel D). This mass is consistent with the peptide (L)LVRY*TKKVPQVSTPTL(V) (1831 amu, from residues 408–423 of the mature albumin sequence, representing a missed peptic cleavage) with an added mass of 162 amu from soman. The parallel fractions from control human plasma do not have peptides of these masses (Figure 1, panels A and C). However, other prominent peaks are present at identical masses in both the control and soman-labeled spectra (compare panels A to B, and C to D), confirming that the fractions are matched with regard to HPLC elution and relative mass spectral

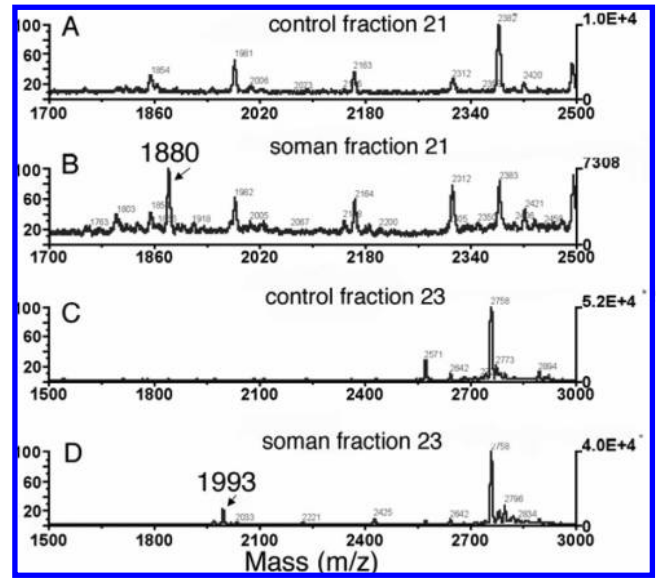


Figure 1. MALDI-TOF mass spectra of peptic peptides from the soman−albumin adduct. Panels A and C, control human plasma digested with pepsin and fractionated by HPLC, but not treated with soman. Panels B and D, human plasma treated with soman before digestion with pepsin and fractionation by HPLC. Soman-labeled albumin peptides of 1880 and 1993 amu include 162 amu from soman.

sensitivity. Tyr411 is present in both peptides (indicated by the star), supporting the proposal that soman binds covalently to Tyr411 of human albumin in plasma. Peptides with an added mass of 162 from soman, but not with an added mass of 78 were found. This indicates that the soman adduct does not lose the pinacolyl group when soman is bound to albumin. Thus, the soman−albumin adduct does not age.

An additional experiment was performed to demonstrate by a second mass spectrometry method that soman was covalently bound to Tyr 411 of human albumin. HPLC fraction 21 containing the soman-labeled albumin peptide at 1880 *m*/*z* was infused into the Q-Trap, quadrupole mass spectrometer. The enhanced mass spectrum showed a peak at 940.7 *m*/*z*, which is consistent with the doubly charged form of the 1880 peptide. This peptide was subjected to collision-induced dissociation. The resulting enhanced product ion spectrum yielded amino acid sequence information consistent with the sequence VRYT-KKVPQVSTPTL, with soman covalently bound to tyrosine (see Figure 2).

The prominent peak at 898.6 *m*/*z* is a doubly charged ion, 42 mass units less than the parent ion at 940.7 *m*/*z*. This is consistent with the loss of the pinacolyl group (84 mass units) from the doubly charged parent to form a doubly charged product retaining methylphosphonic acid. A comparable 42 amu loss from the parent ion was seen in the enhanced product ion spectrum of the LVRYTKKVPQVSTPTL peptide (data not shown). Preferential loss of the pinacolyl group seems to be a characteristic feature of soman-labeled peptides when they are fragmented in the mass spectrometer, as the soman-labeled butyrylcholinesterase peptide also lost the pinacolyl group during the collision process (*32*, *33*).

The 940.7 *m*/*z* parent ion in Figure 2 included the pinacolyl group of soman. This is a significant point because it demonstrates that soman had not lost the pinacolyl group while bound to intact albumin protein or during digestion and HPLC separation. The pinacolyl group only dissociated when the peptide was subjected to collision-induced dissociation in the

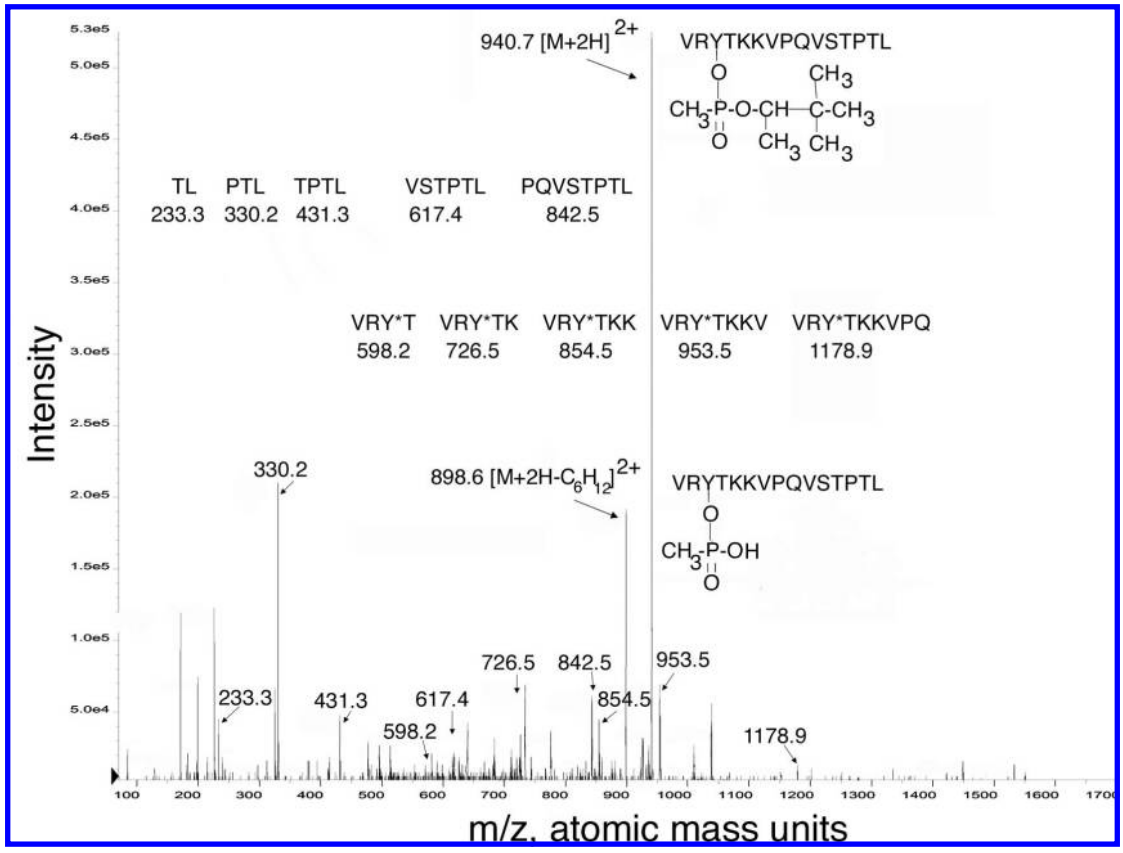


Figure 2. Product ion spectrum of the soman-labeled human albumin peptic peptide. The doubly charged parent ion at 940.7 *m*/*z* yielded fragments consistent with the sequence VRY*TKKVPQVSTPTL, where * indicates soman covalently bound to tyrosine 411. The accession number for human albumin is GI:28592. In this resource, Tyr 411 is given as Tyr 435 because the numbering begins with the 24-amino acid signal peptide.

mass spectrometer. The presence of the pinacolyl group in the parent ion demonstrates that soman did not age when bound to albumin.

At least four separate ion series from the VRYTKKVPQVSTPTL peptide could be extracted from the spectrum in Figure 2. Only the y and b ion series are indicated in the Figure for the sake of clarity.

The peptide [VRY*T]KKV[PQ] was seen as part of the b-ion series. The masses of all five observed fragments were consistent with the indicated amino acid sequence plus 78 amu for the added mass of a methyl phosphonic acid. The only residue in this sequence capable of forming a methyl phosphonic acid adduct is the tyrosine, establishing this as the modified amino acid in the peptide. The presence of a 78 amu added mass on the observed fragments, rather than 162 amu, indicates that the pinacolyl group is dissociated from the peptide, in the collision chamber, prior to the onset of backbone fragmentation.

The peptide [PQ][VS]TP[TL] was seen as part of the y-ion series. The masses of the observed fragments were consistent with only the amino acids, unencumbered with any adduct mass. Thus, the potentially phosphonylated serine was not labeled, supporting the assignment of the tyrosine as the modified amino acid.

The complete amino acid sequence of peptide VRY*TKKV PQVSTPTL was represented by these two ions. These results confirm the conclusion that soman covalently binds to Tyr 411 of human albumin.

3.2. Kinetics for the Phosphonylation of Albumin by Soman and for the Reactivation of Phosphonylated Albumin. 3.2.1. Phosphonylation of Albumin by Soman. Because of the low reactivity of albumin with esters, aryl amides (*1*, *3*), and DFP (*10*), a low soman phosphonylation rate was expected. Therefore, the phosphonylation of albumin by soman was carried out under second-order conditions (*3*, *34*), that is, the initial concentrations of soman, $[S]_0$, and albumin, $[A]_0$, were not very different. As a result, the concentrations of the uncomplexed forms of both reactants varied with time during the reaction.

Albumin (0.78 mM) was phosphonylated by sub and superstoichiometric amounts of racemic soman (0.32 to 1.3 mM) that inhibited albumin's aryl acylamidase activity. Reaction was monitored by following albumin's aryl acylamidase (AAA) activity. Inspection of the inhibition time courses (Figure 3) indicated that less albumin was inhibited by soman than would have been expected from a stoichiometric reaction with the total amount of soman in the reaction. For example, in the presence of a 2-fold excess of soman, 100% inactivation of the AAA activity of albumin was never reached. The titration plot (not shown) for 0.78 mM albumin incubated with different concentrations of soman (0.38, 0.65, 0.81, and 1.30 mM) confirmed this observation. This suggests either that a large part of soman spontaneously hydrolyzed in the nucleophilic Tris/HCl buffer at pH 8.0 or that albumin reacted stereoselectively with specific stereoisomers of soman.

3.2.2. Determination of Fluoride Released during the Reaction of Soman with Albumin. In an effort to decide whether the substoichiometric reaction of soman with albumin was due to a competing spontaneous hydrolysis of soman or to a stereoselective reaction of albumin with soman, ionometric measurements of fluoride released from soman in the presence and absence of albumin were performed (Figure 4). It was found that spontaneous hydrolysis of soman in 60 mM Tris/HCl buffer at pH 8.0 at 25 °C was high. The rate constant for hydrolysis

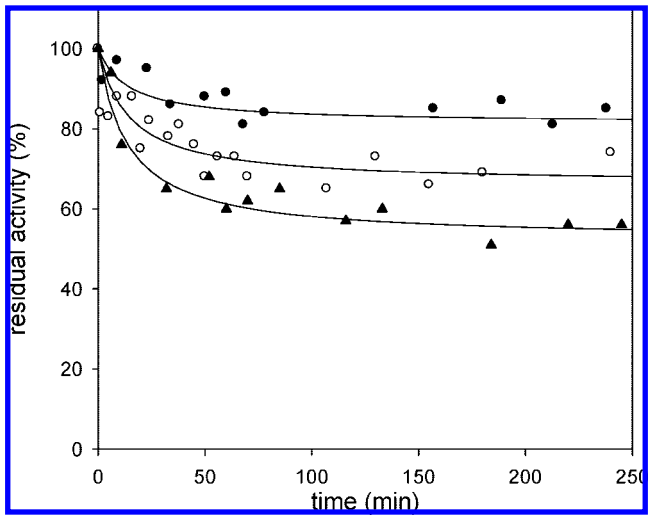


Figure 3. Progressive inhibition of the AAA activity of FAF-HSA ([E] = 0.78 mM) by different concentrations of racemic soman (●, 0.324 mM; ○, 0.520 mM; π, 0.780 mM) in 60 mM Tris/HCl buffer at pH 8.0 at 25 °C. A plot of % residual AAA activity of albumin vs time. The progress curves reach a plateau after consumption of the reactive soman.

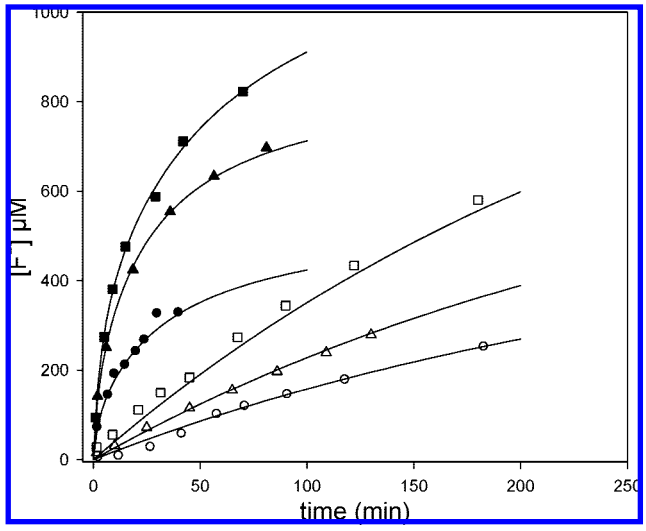


Figure 4. Time course of fluoride ions released from soman, in the presence and absence of FAF-HSA. Soman ($[S]_0$ = ●○, 0.52, ▲△, 0.78, ■□, 1.3 mM) was incubated with albumin ([E] = 0.78 mM) (filled symbols), or soman was incubated by itself through spontaneous hydrolysis (open symbols on continuous curves). Both reactions were in 60 mM Tris/HCl at pH 8.0.

of soman (k_{h0}) was 0.00342 ± 0.00007 min^{-1}. In the presence of albumin, the rate of fluoride release was significantly higher. Semiquantitative analysis of progress curves shows not only that spontaneous hydrolysis of soman occurred at rates comparable to the phosphonylation rates of albumin but also that albumin itself provides a physicochemical environment that enhances the spontaneous hydrolysis of soman. The following is an example of this semiquantitative analysis.

The filled squares in Figure 4 describe the release of fluoride after mixing 1.3 mM soman with 0.78 mM FAF-HSA. After 100 min of incubation, 0.3 mM albumin was inhibited (see Figure 3) so that the phosphonylation of albumin accounts for 0.3 mM of released fluoride. Over this time period, about 0.9 mM fluoride was released. Spontaneous hydrolysis of 1.3 mM soman (□ in Figure 4) accounts for about 0.35 mM of released fluoride. This leaves about 0.25 mM fluoride unaccounted for. Thus, the release of this extra fluoride suggests that the hydrolysis of soman at pH 8.0 is faster in the presence of albumin than in buffer alone. However, dephosphonylation of the Tyr411 adduct is far too slow (0.0044 ± 0.0008 h^{-1}; see section 3.2.5) to account for this albumin-dependent hydrolysis of soman. Thus, this suggests that this catalysis takes place at a different site. However, only one soman adduct on albumin was detected by MALDI-TOF. This finding is consistent with reports on the phosphonylation of bovine serum albumin by FP-biotin (*18*) and the phosphorylation of human serum albumin by diisopropylfluorophosphate (*9*). Therefore, it may be hypothesized that albumin-mediated hydrolysis of soman occurs via a mechanism that does not involve a covalent intermediate. Nevertheless, this catalytic effect is extremely weak, about 2 times higher than spontaneous hydrolysis, and may be tentatively regarded as a nonenzymatic, physicochemical interfacial reaction. However, we cannot rule out the existence of a second active center.

3.2.3. Is There Enantioselectivity in the Reaction of Albumin with Soman? Racemic soman is an equimolar mixture of four stereoisomers. Though experiments on the release of fluoride argue against the enantioselectivity of albumin for certain soman isomers, the possibility that albumin's reaction with soman is stereoselective was tested directly using ^{31}P NMR. Each of the intact isomers of soman (Ps− and Pr−) generates a doublet in ^{31}P NMR (Figure 5, panel A). Soman completely hydrolyzed in 5 N sodium hydroxide gives methylphosphonate (MP) (Figure 5, panel B); the hydrolysis of soman at pH 8.0 gives pinacolyl methylphosphonate PMP (Figure 5, panel D) (*35*).

The reaction between 1.3 mM racemic soman and 0.78 mM albumin was monitored over 24 h by recording ^{31}P NMR spectra. Initial spectra showed the progressive disappearance of the intact soman peaks with the concomitant appearance of three broader resonance peaks, of 80 Hz line width (Figure 5, panel C). After 2 h of incubation, signals for intact soman had vanished. At this point in the reaction, the spectrum of the products was compared to a spectrum of methylpinacolyl phosphonate (MPP). MPP, created under mild conditions of hydrolysis, showed a peak at 23.74 ppm. That peak was unaffected by the presence of albumin (Figure 5, panel D). The most prominent peak in the soman/albumin reaction mixture (the 23.74 ppm peak, f, in Figure 5, panel C) can therefore be assigned to MPP. For longer reaction times (up to 24 h), the MPP peak increased at the expense of the other two peaks in Figure 5, panel C. This strongly suggests that peaks e and d (at 25.59 and 27.56 ppm) correspond to the Ps− and Pr− covalent adducts of soman bound to albumin and that there is a slow, spontaneous release of MPP from the of phosphonlyated albumin. Such a release can be described as reactivation.

The fact that after 2 h of reaction between albumin and soman, adduct peaks d and e have the same intensity indicates that albumin reacted with the same probability with both the Ps− and Pr− soman enantiomers. Thus, there is no enantiomeric preference of albumin for the soman isomers.

3.2.4. Effect of MPP on Phosphonylation of Albumin by Soman. Because a large part of soman was hydrolyzed into MPP in Tris buffer at pH 8.0 during the time course of the phosphonylation reaction (Figure 5, panel C), we tested the hypothesis that MPP could compete with soman for noncovalent binding to the Tyr411 site and thereby slow down the phosphonylation reaction. We found that phosphonylation of (0.75 mM) albumin by soman (1.3 mM) in the presence of 1.3 mM MPP caused no change in the progressive inhibition of the AAA activity of albumin. Thus, MPP does not compete with soman.

3.2.5. Spontaneous Reactivation of Soman-Inhibited Albumin Monitored by ^{31}P NMR and MALDI-TOF. Fluoride has been shown to promote dephosphonylation of albumin (*36*).

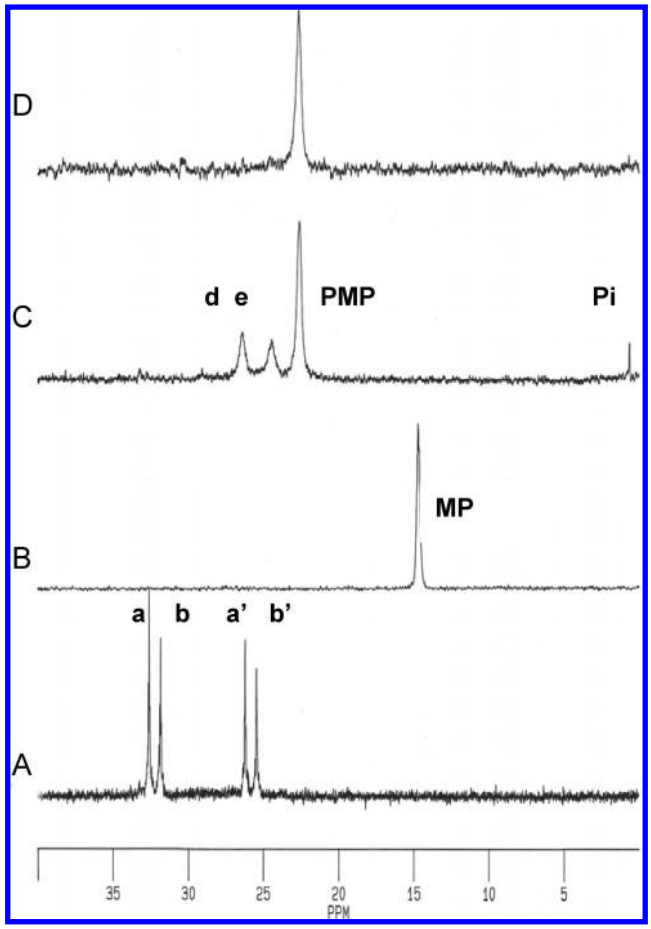


Figure 5. ^{31}P NMR spectra of soman in the presence and absence of albumin. Panel A shows 2 mM racemic soman in dimethylsulfoxide-d_6. The four stereoisomers are indistinguishable. The four resonance peaks correspond to the two doublets of the two pairs of diastereoisomers (35). Panel B shows methyl phosphonate (MP) (a product of soman hydrolysis in 5 N NaOH). Panel C shows 1.3 mM racemic soman after reaction (2 h) with 0.78 mM albumin, in 60 mM Tris/HCl at pH 8; d and e correspond to the P_R and P_S adducts of albumin, and f is the product of the spontaneous hydrolysis of soman and methyl pinacolyl phosphonate (MPP). Panel D shows 1.3 mM methyl pinacolyl phosphonate (MPP) in the presence of 1.3 mM albumin. The observed peak is that of uncomplexed MPP (35).

However, because of the low nucleophilicity of fluoride ion, it seems unlikely that reactivation was mediated by the fluoride ions released during the phosphonylation step ($[F^-] \leq 1.3$ mM for the highest soman concentration used). Following the reactivation rate by measuring the recovery of the AAA activity was not accurate; therefore, ^{31}P NMR spectral kinetics was used instead.

FAF-HSA (1.3 mM) was inhibited by 1.3 mM soman (10% isopropanol, final). We followed the decrease of the integrated area of the NMR peaks corresponding to the soman−albumin adduct (peaks d + e in Figure 5, panel C) from 10 to 60 h (Figure 6). Because no soman was left after 10 h of incubation, because of its spontaneous hydrolysis (S → S″ + F^-) and to its reaction with albumin (see Scheme 1), the change in the concentration of the soman−albumin conjugate (A−S′) was due only to reactivation (k_r). It was assumed that the process was uncompromised and followed pseudo-first-order kinetics (eq 1):

$$[A-S']_t = [A-S']_{10h} \cdot e^{-k_r \cdot (t-10)} \quad (1)$$

The relative intensity at $t = 10$ h was 0.54 ± 0.01; this is equivalent to 0.700 ± 0.015 mM of conjugate. Fitting the data from Figure 6 to eq 1 provided the reactivation constant, k_r = 0.0044 ± 0.0008 h^{-1} = 0.000073 ± 0.000013 min^{-1} = 0.74 ± 0.13 $week^{-1}$. Thus the half-time for decay of the soman−albumin adduct at pH 8.0 and 25 °C is about 1 week.

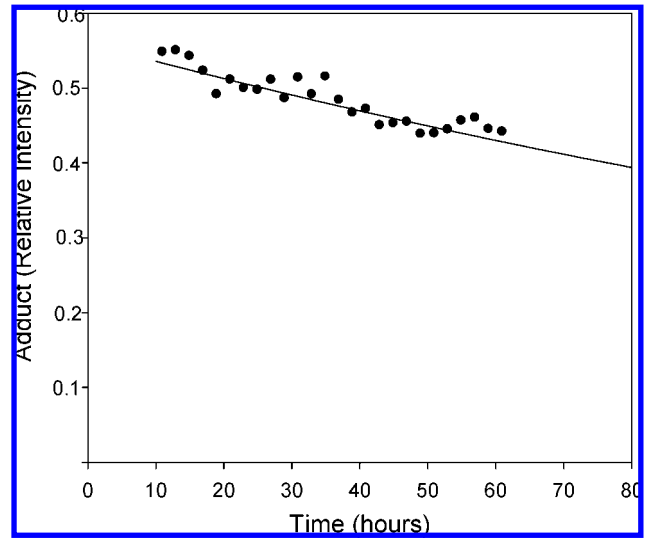
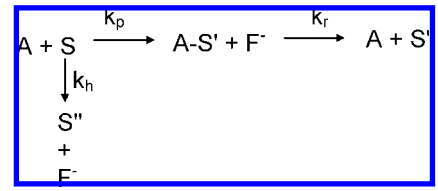


Figure 6. Reactivation kinetics by ^{31}P NMR. Each point represents the integrated area of the NMR peaks (d + e). The areas, in turn, correspond to the relative amounts of adduct. The line is from a fit of the data to eq 1.

Scheme 1



The stability of the soman−albumin adduct was also measured by mass spectrometry. Soman-labeled albumin was digested with pepsin to release labeled and unlabeled Tyr411 peptides after various periods of incubation. The peptide masses and the area of each peak were determined by MALDI-TOF in reflector mode. Figure 7A shows a scan, after 24 h, where the highest peaks at 1992.1 and 1879.0 have soman bound to Tyr411 in peptides LVRYTKKVPQVSTPTL and VRYTKKVPQ-VSTPTL. At a later time point after the adduct had been incubated at pH 7.4 and 22 °C for 768 h, the unlabeled peptides at 1717.0 and 1830.1 have a higher intensity than the labeled peptides (Figure 7B). The % label on Tyr411 was calculated from cluster areas and plotted in Figure 7C as a function of time. The half-life for decay of the soman−albumin adduct at pH 7.4 and 22 °C was 20 days. The time course in Figure 7C is first order for nearly two half-lives, supporting the proposal that reactivation is a simple first order process. The difference in rate constants obtained from the mass spectrometry and the NMR experiments is consistent with expectation for the difference in pH.

3.2.6. Progressive Binding of Soman to Tyr411 of Albumin Monitored by ^{31}P NMR Kinetics. The complete reaction path of albumin (A) with soman (S) can be depicted by Scheme 1, which includes the competing spontaneous hydrolysis of soman, second-order phosphonylation of albumin, followed by hydrolytic dephosphonylation (reactivation of the AAA activity of albumin).

In Scheme 1, k_p is the rate constant of phosphonylation, k_r is the rate constant of dephosphonylation (spontaneous reactivation of phosphonylated albumin A-S′), and k_h is the rate of the

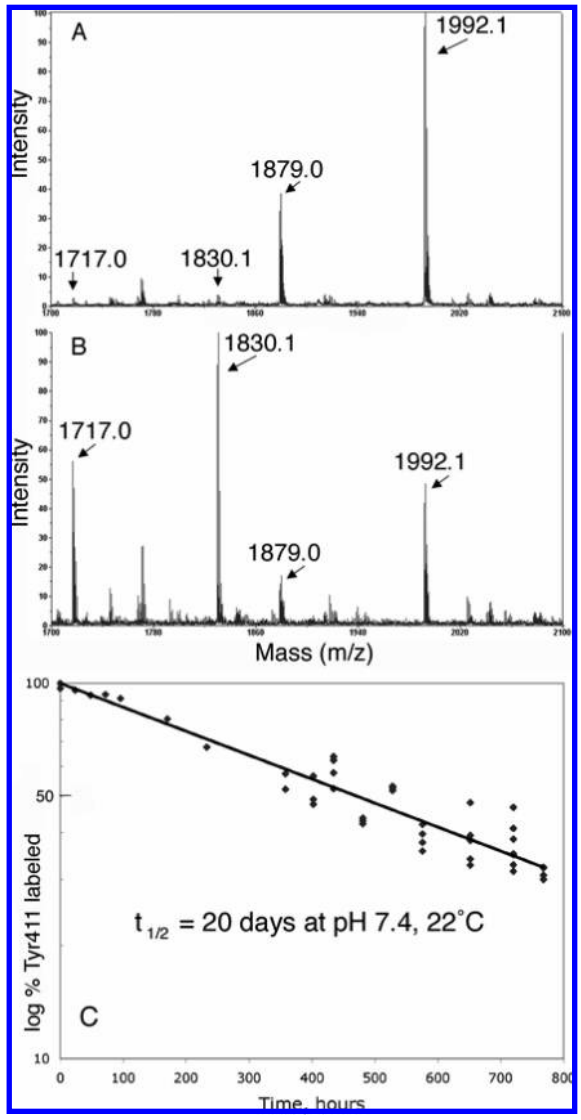


Figure 7. Stability of the soman−albumin adduct calculated from mass spectrometer data. Panels A and B are MALDI-TOF spectra of pepsin-digested, soman-labeled human albumin after 24 and 768 h incubation, respectively, at pH 7.4 and 22 °C. Panel C is a plot of % Tyr411 labeled by soman, as a function of time of incubation of the soman−albumin adduct. The soman−albumin adduct decays with a half-life of 20 days.

spontaneous hydrolysis of soman. F^- is the fluoride leaving group and S″ the hydrolysis product, methyl pinacolyl phosphonate (MPP). It follows then that the concentration of all species as a function of time is described by a system of differential equations (eq 2) as sollows:

$$\begin{aligned}
\frac{d[A]}{dt} &= -k_p \cdot [A][S] + k_r \cdot [AS'] \\
\frac{d[S]}{dt} &= -k_p \cdot [A][S] + k_h \cdot [S] \\
\frac{d[AS']}{dt} &= -\frac{d[A]}{dt} \\
\frac{d[S'']}{dt} &= -k_r \cdot [AS'] + k_h \cdot [S] \\
\frac{d[F^-]}{dt} &= -\frac{d[S]}{dt}
\end{aligned} \tag{2}$$

Because the concentration of soman and albumin are of the same order of magnitude and because the rates of spontaneous hydrolysis of soman and phosphonylation of albumin are of the same order of magnitude, it is not possible to make assumptions allowing a simple integration of the system. However, a numerical solution of the system can be obtained using mathematical software such as Mathematica.

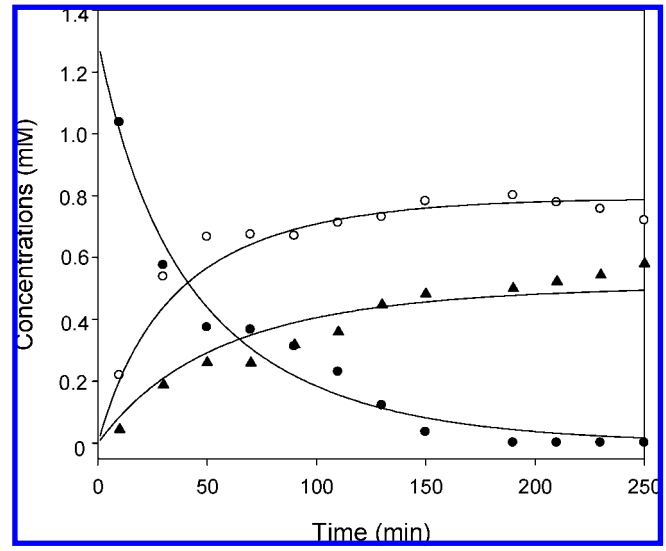


Figure 8. Time course of the reaction between 1.3 mM soman and 1.3 mM albumin monitored by ^{31}P NMR: ●, soman; ○, adduct; ▲, MPP. The points were measured data. The lines are from numerical fitting of the data to eq 2.

Following the residual AAA activity of albumin allows only the measurement of d[A]/dt = −d[AS′]/dt, while measuring the release of F^- gives only d[F^-]/dt = d[S]/dt. However, following the reaction kinetics by ^{31}P NMR gives d[S]/dt = −d[F^-]/dt, d[AS′]/dt = −d[A]/dt, and d[S″]/dt in one single experiment (Figure 8). k_r was measured separately under the same experimental conditions ($k_r = 0.0044 \pm 0.0008$ h^{-1}; see section 3.2.4 and Figure 6).

Fitting the numerical solution of the differential equation system against ^{31}P NMR data gave $k_p = 15 \pm 3$ M^{-1} min^{-1} and $k_h = 0.0076 \pm 0.005$ min^{-1}. Thus, the rate constant for spontaneous hydrolysis of soman in the presence of albumin (k_h) is about 2 times the rate for the spontaneous hydrolysis of soman in the absence of albumin ($k_{h0} = 0.00342 \pm 0.00007$ min^{-1}, from section 3.2.2). This value for k_h is in agreement with the estimate for k_h made from the measurement of [F^-] release, as described in section 3.2.2.

3.3. Molecular Modeling. The crystal structure of HSA with numerous ligands has been described (*5*). A comparison between these structures shows that Val433 located in the pocket containing Tyr411 might adopt two different conformations depending on the occupant of the pocket. These alternate conformations change the diameter of the pocket and may affect the binding of soman in the pocket. Therefore, we docked soman with both representative crystal structures: 2bxd displaying a narrower pocket in absence of ligand and 2bxh displaying a wider pocket that develops when indoxyl sulfate is bound. The four diastereoisomers of soman were docked in both structures.

3.3.1. Noncovalent Docking by Autodock. Key values from the noncovalent docking results are summarized in Table 1. All four stereoisomers of soman bind better to HSA when the pocket is narrowed by the conformational change of Val433 (2bxd). A narrower pocket provides more stabilizing van der Waals interactions between soman and the residues of the pocket. Therefore, it is expected that the actual conformation of HSA when in complex with soman is similar to that of 2bxd. For 2bxd, the estimated affinity constants for all four stereoisomers

Table 1. Non Covalent Docking of Soman in the Tyr411 Pocket of HSA Performed by Autodock

soman isomer	HSA pdb structure	docked energy (kcal.mol^{-1})	estimated K_d (μM)
P_SC_R	2bxd	−5.69	148
	2bxh	−5.56	167
P_SC_S	2bxd	−5.76	115
	2bxh	−5.48	206
P_RC_R	2bxd	−5.69	147
	2bxh	−5.50	176
P_RC_S	2bxd	−5.73	128
	2bxh	−5.42	226

are virtually identical: $K_d = 134 \pm 16$ μM (Table 1). The pocket seems to be large enough to accommodate all enantiomers of soman (Figure 9). Soman binds with the pinacolyl group stacked against the disulfide bridge formed by Cys392-Cys438. The pinacolyl group makes many hydrophobic contacts with the side chains of Val433, Ile388, Ala449, Phe395, Phe403 and the main chain of Gly434. The binding conformation of soman is largely dictated by its carbon stereocenter. Indeed, P_SC_S and P_RC_S display the same binding conformation (rmsd = 0.07 Å), except that the positions of the fluoride and phosphonate oxygen are inverted as a result of the difference in stereochemistry at the phosphorus. The same applies to the P_SC_R and P_RC_R stereoisomers (rmsd = 0.13 Å). This inversion between the fluoride and phosphonate oxygen does not cost much energy of binding because of the lack of specific interactions between these atoms and residues of the pocket. The phosphorus atom is separated from the hydroxyl of Tyr411 by a distance ranging from 6.80 Å (C_R enantiomers) to 7.24 Å (C_S enantiomers). This distance is not suitable for a nucleophilic attack of Tyr411 on the phosphorus and explains the slow reactivity of soman toward HSA. It looks like soman may randomly react with Tyr411 on its way in/out of the pocket.

3.3.2. Noncovalent Docking by Gold. In order to check the dependency of the results on the software, to test the robustness of the models obtained by Autodock, and also to generate as many conformers as possible, we decided to repeat the docking simulations using another software, Gold, version 3.1. Gold gave us the possibility of using two scoring functions, Goldscore (force-field-based like Autodock) and Chemscore (empirical, i.e., optimized on a test set of protein–ligand complexes). We performed the docking simulations using both the 2bxh and the 2bxd protein structures. Our results did not show any significant difference between the structures; therfore, we describe here only the results obtained with the minimized 2bxh protein.

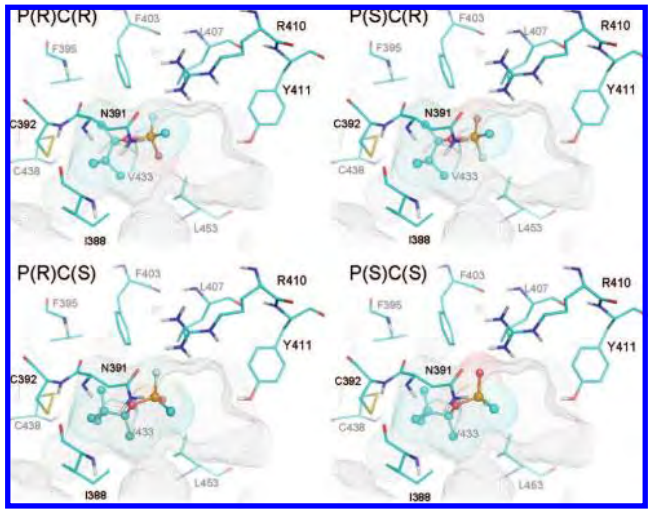


Figure 9. Models of soman noncovalently docked into albumin. The various stereoisomers of soman were docked in the estero-amidase active center (Tyr411) of HSA using Autodock software.

We docked the four enantiomers of soman with the minimized 2bxh protein using Gold/Goldscore. The best conformers obtained were similar to the ones obtained by Autodock. The pinacolyl moiety is located at essentially the same place obtained by Autodock (Figure 9); the difference is only a small translation of Tyr411 in the direction of the phosphorus atom (P-OH_{Y411} distances vary from 6.37 to 6.67 Å). As described for Autodock, the rotation of the P (O, F, Me) moiety is relatively free; this is not surprising because of the lack of specific interactions of these atoms with residues of the active site pocket. Again, the phosphorus atom is at a position that does not permit easy phosphonylation of Tyr411. The scores obtained were in the same range for all enantiomers: $P_SC_S = 39.93$, $P_SC_R = 40.09$, $P_RC_R = 41.28$, and $P_RC_S = 38.16$. We obtained very similar results in terms of conformers and energies when we used Autodock or Gold with either 2bxh or 2bxd, which clearly shows the robustness of the models in Figure 9.

The Chemscore function is empirical. It can potentially generate different binding modes because contributions of intermolecular interactions are not the same as those with Goldscore or Autodock. The docking using Gold/Chemscore gave different kinds of conformers than Goldscore, but with very small changes in energy. Within the different clusters, some models displayed a phosphorus atom close to Tyr411 (P-OH_{Y411} distances: $P_RC_R = 4.33$ Å, $P_RC_S = 3.51$ Å, $P_SC_S = 3.98$ Å, and $P_SC_R = 3.41$ Å). The enantiomers with P_R configuration have a P-F in a favorable position for a nucleophilic attack (OH_{Y411}-P-F angle: $P_RC_R = 155°$, $P_RC_S = 138°$, $P_SC_S = 42°$, and $P_SC_R = 45°$). Chemscore docking simulation generated a prephosphonylation complex (fluorine opposite OH_{Y411} and hydrogen bonding between P = O and OG_{S489}) for the two P_R enantiomers. These results suggest that the prephosphonylation step would be energetically more favorable for the P_R than for the P_S enantiomers. To compare these models with the previous ones, we rescored the prephosphonylation complexes with conformers P_RC_R and P_SC_S by the Goldscore function and obtained 10.78 and 27.07, respectively. Compared to the Gold/Goldscore results (41.28 and 38.16), the rescoring clearly indicates that the ligand positions in the prephosphonylation complexes are not energetically favorable. Therefore, the Chemscore results do not contradict the previous conclusions obtained from the Autodock and Gold/Goldscore dockings.

These docking results may explain why the reactivity of soman with HSA is so poor. First, the binding affinity is weak $\langle K_d \rangle = 134$ μM. Second, the phosphorus does not bind in a position that is favorable for reaction with Tyr411. The docking results also suggest that a higher bimolecular rate constant is probable in the case of P_R enantiomers. But because of the poor reactivity and the limited measurement accuracy, an increased reactivity of the P_R enantiomers was not experimentally observed.

3.3.3. Covalent Docking by Gold. The noncovalent docking of soman to the HSA protein showed a low enantioselectivity. In order to confirm this finding, we covalently docked the four enantiomers of soman to the 2bxh form of albumin and compared energies (or scores). We used the software Gold, version 3.1, which permits covalent docking. As done before, dockings were scored by the functions Goldscore and Chemscore. To discriminate the conformers obtained by Gold/Goldscore or Gold/Chemscore dockings, we calculated the energies of soman−protein complexes using mopac-pm3, a

Table 2. Covalent Docking of Soman in HSA Performed by Gold[a]

soman isomer	Goldscore/ΔH_f	Chemscore/ΔH_f
P_SC_R	11.68/489.8	15.95/519.3
P_SC_S	16.88/483.2	17.43/514.7
P_RC_R	15.67/529.0	16.46/497.8
P_RC_S	12.88/480.4	14.55/539.8

[a] Scores (Goldscore and Chemscore) have no units. Enthalpies of formation (ΔH_f) are in kcal·mol^{-1}.

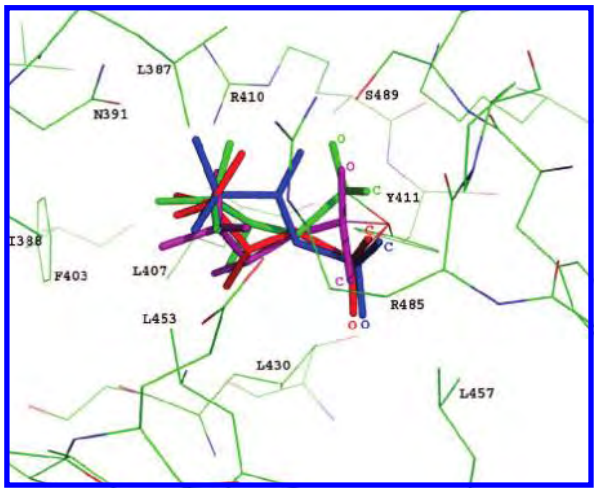


Figure 10. Models of soman covalently docked into albumin. Various enantiomers of soman were docked into Tyr 411 of HSA (form 2bxh) using Gold software, version 3.1. P_SC_S (blue), P_SC_R (red), P_RC_R (magenta), and P_RC_S (green).

semiempirical method, and selected those with the lowest ΔH_f. The results are summarized in Table 2.

The four models are superimposed in Figure 10. The position of the tertiary butyl group is well conserved for all enantiomers (it points to Leu387, Ile388, Asn391, and Phe403), while the *P* = O points to Leu430 and Leu457 for P_SC_R or P_SC_S enantiomers, Ser489 (hydrogen bonding with OH) for P_RC_R, and R410 for P_RC_S. The conformers P_SC_S and P_SC_R are nearly perfect mirror images, the only difference being the position of the CH_3 bond to the asymmetric carbon that points to Leu430 for C_R and Arg410 for C_S. In the case of P_R, there are more differences between the two enantiomers because the P_RC_R enantiomer is the only one that allows hydrogen bonding with Ser489 without having too many disfavored van der Waals interactions. (Arg410 constrains the position of the CH_3 bond to C*.) The mopac-pm3 ΔH_f values are in the same order of magnitude (~490 ± 10 kcal.mol^{-1}), showing no thermodynamic enantioselectivity.

4. Discussion

4.1. Roles for Albumin and Albumin/Soman Adducts in the Monitoring of and Protection against Soman Exposure. 4.1.1. Scavenging OP. The reaction of albumin with soman was found to be slow (k_p = 15 ± 3 M^{-1} min^{-1} at pH 8.0). This is in agreement with the slow rate of reaction of albumin with the nerve agent simulant, DFP (75 M^{-1} min^{-1} at pH 8.2) (*10*). Combined with the high concentration of albumin present in plasma (0.6 mM), the apparent pseudo-first-order rate constant for the reaction of albumin with soman would be 0.009 min^{-1} ($t_{1/2}$ = 77 min). Thus, despite its high concentration in blood, stoichiometric scavenging of soman by albumin is not expected to play a major role in protection against acute poisoning. Regardless of the low reactivity, significant amounts of OPs are bound to albumin upon in vivo exposure. For example, when mice were injected with small amounts of the organophosphonate, FP-biotin (10-fluoroethoxyphosphinyl-*N*-biotinamido pentyldecanamide), amounts that produced no toxic signs, albumin was the most abundantly labeled protein in plasma (*46*), making it the dominant stoichiometric scavenger. In vitro pretreatment of plasma with a variety of organophosphates (malaoxon, paraoxon, chlorpyrifos oxon, methyl paraoxon, dichlorvos, diisopropylfluorophosphate, diazoxon, and echothiophate) reduced the subsequent reaction of FP-biotin with albumin, indicating that all of these other OPs also react with albumin (*46*). This strongly suggests that albumin will also be a scavenger for these OPs in vivo.

Although albumin displays a low reactivity toward OPs compared to that of cholinesterases and other known secondary biological targets of OPs (*37*), the large quantity of albumin in blood circulation and lymph may provide a substantial reservoir for sequestering OPs. We also found that phosphonylated albumin slowly self-reactivates. Though the catalytic OPase activity is associated with HSA, and Tyr411 is slow and thus toxicologically insignificant, it may be hypothesized that mutants of albumin capable of hydrolyzing OPs at high rate could be used for the detoxification of soman in vivo.

4.1.2. Biomarker. Phosphonylated albumin may prove to be a useful biological marker of soman exposure (*11*). The sensitive mass spectrometry methods used by Black et al. (*11*) and further developed in the present article could prove useful for the diagnosis of soman exposure. In particular, it has been shown that soman tyrosine adducts can be detected at least up to 7 days post exposure in guinea pigs (*38*). Advantages of a soman−albumin biomarker include its stability and the absence of aging. The half-life for spontaneous reactivation is estimated to be about 1 week at pH 8.0 and 25 °C, and 20 days at pH 7.4 and 22 °C, making the soman−albumin adduct suitable for retrospective studies. The absence of aging means that soman exposure can be unambiguously identified because the soman−albumin adduct does not lose the diagnostic pinacolyl group. Our finding that soman−albumin adducts do not age confirms the reports that there is no loss of alkoxy groups from soman−, sarin−, or DFP−albumin adducts (*11*, *19*, *36*). In addition, it has recently been shown that OP−albumin conjugates do not appear to be reactivatable by oximes used as antidotes of OP poisoning. Therefore, the detection of Tyr411 adducts in plasma may be possible, even though individuals exposed to OPs have received effective oxime therapy (*38*).

Another potential method of monitoring for soman exposure is through the use of antibodies. The information presented in this work raises interesting possibilities regarding the generation of antibodies to the soman−albumin adduct. The noteworthy aspect of the soman−albumin adduct in this regard is that Tyr411 is located on the surface of albumin. A soman−albumin adduct at Tyr411 therefore becomes a viable target for antibodies, unlike the soman adducts of cholinesterases, which are buried in a deep gorge well below the enzyme's surface. In addition, the surface location for the soman−albumin adduct suggests that people exposed to soman might normally produce antibodies against the soman−albumin adduct. This in turn suggests the possibility of monitoring exposure to soman by looking for those antibodies, long after the exposure incident has passed and long after the antigen has disappeared.

4.2. Perspectives: Would Mutant Albumins Be Good OP Scavengers? 4.2.1. Stoichiometric Scavenging. The bimolecular rate constant for the phosphonylation of albumin, k_p, 15 M^{-1} min^{-1}, is about 6 × 10^6 times slower than that for the inhibition of acetylcholinesterase and butyrylcholinesterase by

soman ($\sim 9 \times 10^7$ M^{-1} min^{-1}). Because the concentration of soman in the most severe cases of poisoning would be far less than that of albumin in plasma, for example, <11 nM (*39*), the reaction between albumin and soman would be pseudo-first-order. The pseudo-first-order rate expression can be rearranged (eq 3) so that the time, *t*, needed for a stoichiometric scavenger to reduce a concentration of a toxicant such as soman from $[S]_0$ to $[S]_t$ can be easily determined.

$$t = \frac{\ln([S]_0/[S]_t)}{k_p \cdot [E]} \quad (3)$$

Since the concentration of albumin in plasma is about 0.6 mM, it would take about 8.5 h to reduce the soman concentration by 100-fold (Ln $[S]_0/[S]_t = 4.6$). Because, red blood cell acetylcholinesterase, plasma butyrylcholinesterase, and plasma paraoxonase react much faster than albumin with soman, the contribution of albumin to soman detoxification would take place only after the saturation of these enzymes (*36*).

4.2.2. Catalyic Scavenging. If we assume that the turnover of soman by albumin fits a Michael mechanism with a high K_m, then phosphonylation could appear to be second-order under our experimental conditions. If in addition, $k_r << k_p$, which is the case for the soman/albumin turnover, then k_{cat}/K_m is equal to k_p. Under these conditions, k_p defines the catalytic efficiency. Thus, the catalytic efficiency for the hydrolysis of soman by albumin, at Tyr411, is 15 M^{-1} min^{-1}. This value is about 4 to 5 orders of magnitude lower than that for the hydrolysis of soman by *Pseudomonas diminuta* phosphotriesterase (6×10^5 M^{-1} min^{-1}) (*40*) or allozyme Q192 of human paraoxonase (2.6×10^6 M^{-1} min^{-1}) (*41*). Eq 4 gives the time needed for a catalytic scavenger to drop the concentration of a toxicant from $[S]_0$ to $[S]_t$.

$$t = \frac{\ln([S]_0/[S]_t)}{(k_{cat}/K_m) \cdot [E]} \quad (4)$$

Thus, taking the estimated k_{cat}/K_m value, the time needed for the catalytic activity of albumin (0.6 mM) to decrease the soman concentration in blood by 100-fold would be about 9 h. It is therefore obvious that the reaction of soman at Tyr411 of HSA does not contribute significantly to the catalytic turnover of soman molecules in plasma. However, the in vitro observation that pure FAF-HSA promotes the hydrolysis of soman at a second site ($k_h = 0.0076$ min^{-1}) with a rate 100 times higher than the turnover at Tyr411 is important. The in vivo toxicological relevance of this chemical activity has to be demonstrated, but if proved, this process could contribute to the degradation of soman and other nerve agents in human plasma.

4.2.3. Mutant Albumin. Could mutants of recombinant human albumin be made into productive stoichiometric or catalytic scavengers for prophylaxis and treatment of OP poisoning? HSA is a nonglycosylated protein. Scaled-up production of recombinant human albumin (rHSA) in *Pichia pastoris* has been made possible for pharmaceutical purposes (*42*). This product exhibits good pharmacokinetics and biodistribution (*43*). Thus, rHSA would appear to be an ideal candidate for protein engineering.

To address the scavenger issue of rHSA, we have to first determine how much improvement in reactivity would be desired. As a reference, the reactivity of the currently accepted OP scavengers, serum butyrylcholinesterase and red cell acetylcholinesterase, were used. Comparison was made on the basis of the first-order rate constants. For the reaction of natural HSA with soman in plasma, the apparent pseudofirst order rate constant, $k_p \cdot [E]$, is 0.009 min^{-1}. The sum of the first-order reaction rate constants for the reaction of soman with erythrocyte AChE and plasma BuChE is 4.5 min^{-1}. This number was arrived at by taking the concentration of AChE in blood to be about 2.5 nM, the concentration of BuChE to be 50 nM, and k_2/K_d to be about 9×10^7 M^{-1} min^{-1} for both enzymes. Thus, in blood, cholinesterases react with soman about 500 times faster than albumin does.

For an injected, mutated albumin to be used as a stoichiometric scavenger, it should react at least 500 times faster than the endogenous albumin to be competitive with the cholinesterases. There is a limit to the amount of plasma-derived albumin that can be put into the human body, that is, 25 g/100 mL (Albu Rx25) by slow infusion or 500 mg in 2 mL by intravenous injection. Injected into a human of 70 kg (3 L of plasma), 500 mg would give a final plasma concentration of mutant albumin of 166 mg/L (2.55×10^{-6} M). To react faster than cholinesterases, the bimolecular rate constant of this amount of mutant albumin should be at least 1.8×10^6 M^{-1} min^{-1}. Compared to endogenous albumin (15 M^{-1} min^{-1}), this is an efficiency improvement of about 5 orders of magnitude. Though there are few examples of evolved enzymes, showing enhancement of such a magnitude, site-directed mutagenesis and/or directed evolution can lead to dramatic improvement in the catalytic activity of enzymes. Improvements of 4 orders of magnitude for the glyphosate tolerance protein (*44*) and 5 orders of magnitude for α-lytic protease (*45*) have been reported. Therefore, improving the reactivity of albumin against OPs for the purpose of making a stoichiometric scavenger would be a challenge, but it is possible.

In addition, the observed catalysis of soman hydrolysis on the albumin surface at a site different from Tyr 411 could also be improved. Work is in progress to identify this area on the albumin surface. Thus, stoichiometric and catalytic bioscavengers based on modified human albumins have to be considered as possible candidates.

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Mass spectrometry identifies covalent binding of soman, sarin, chlorpyrifos oxon, diisopropyl fluorophosphate, and FP-biotin to tyrosines on tubulin: A potential mechanism of long term toxicity by organophosphorus agents

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ABSTRACT

Chronic low dose exposure to organophosphorus poisons (OP) results in cognitive impairment. Studies in rats have shown that OP interfere with microtubule polymerization. Since microtubules are required for transport of nutrients from the nerve cell body to the nerve synapse, it has been suggested that disruption of microtubule function could explain the learning and memory deficits associated with OP exposure. Tubulin is a major constituent of microtubules. We tested the hypothesis that OP bind to tubulin by treating purified bovine tubulin with sarin, soman, chlorpyrifos oxon, diisopropylfluorophosphate, and 10-fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide (FP-biotin). Tryptic peptides were isolated and analyzed by mass spectrometry. It was found that OP bound to tyrosine 83 of alpha tubulin in peptide TGTYR, tyrosine 59 in beta tubulin peptide YVPR, tyrosine 281 in beta tubulin peptide GSQQYR, and tyrosine 159 in beta tubulin peptide EEYPDR. The OP reactive tyrosines are located either near the GTP binding site or within loops that interact laterally with protofilaments. It is concluded that OP bind covalently to tubulin, and that this binding could explain cognitive impairment associated with OP exposure.

1. Introduction

Acute toxicity from organophosphorus poisons (OP) is mainly due to inhibition of acetylcholinesterase [1]. However, low dose exposure that causes minimal inhibition of AChE and no obvious cholinergic symptoms has been linked to memory loss, sleep disorder, depression, learning and language impairment, and decreased motor skills in humans [2–4]. Rats treated with low doses of chlorpyrifos have behavioral deficits in a water–maze hidden platform task and in prepulse inhibition [5]. The mechanism to explain cognitive deficits from low dose exposure is thought to be inhibition of fast axonal transport [5]. Axonal transport was impaired in sciatic nerves isolated from chlorpyrifos treated rats [5,6]. Transport of nutrients to nerve endings is accomplished via microtubules that serve as the highway on which kinesin molecules

Abbreviations: amu, atomic mass units; CPO, chlorpyrifos oxon; DFP, diisopropyl fluorophosphate; FP-biotin, 10-fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide; FPB, FP-biotin; OP, organophosphorus poisons; CID, collision induced dissociation.

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carry their cargo [7]. When microtubule function is disrupted, neurons lose viability. Microtubules are polymers of alpha and beta tubulin. Prendergast et al. have shown that polymerization of tubulin is inhibited by low doses of chlorpyrifos and diisopropyl fluorophosphate (DFP) [8]. The goal of the present work was to identify the amino acid residues modified by reaction of tubulin with OP. Mass spectrometry identified four covalent binding sites, all of them tyrosines.

2. Materials and methods

2.1. Materials

Bovine tubulin (TL238) >99% pure, isolated from bovine brain, was from Cytoskeleton, Inc. (Denver, CO). This tubulin preparation contains both alpha and beta-tubulin. Chlorpyrifos oxon (MET-674B) was from Chem Service Inc. (West Chester, PA). 10-Fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide (FP-biotin) was custom synthesized in the laboratory of Dr. Charles M. Thompson at the University of Montana, Missoula, MT [9]. Diisopropylfluorophosphate (D0879) was from Sigma–Aldrich (St. Louis, MO). The nerve agents sarin and soman were from CEB (Vert-le-Petit, France). Sequencing grade modified porcine trypsin (V5113) was from Promega (Madison, WI). Slide-A-Lyzer 7K dialysis cassettes (No. 66370) and ImmunoPure immobilized monomeric avidin (#20228) were from Pierce Biotechnology Inc. (Rockford, IL).

2.2. OP-labeled tubulin tryptic peptides

Bovine tubulin (2 mg/ml) was dissolved in either 50 mM ammonium bicarbonate pH 8.3 or in 80 mM PIPES, 0.5 mM EGTA, 0.25 mM $MgCl_2$ buffer pH 6.9, or in 10 mM Tris–Cl pH 8.0. The 0.5 ml of 2 mg/ml tubulin (40 μM) was treated with a 20-fold molar excess of FP-biotin dissolved in dimethyl sulfoxide, or a 200-fold molar excess of diisopropyl fluorophosphate (DFP), or a 20-fold molar excess of chlorpyrifos oxon (CPO) dissolved in dimethyl sulfoxide, or a 5-fold molar excess of soman and sarin dissolved in isopropanol. The reaction mixtures were incubated at 37 °C for 16–24 h. The proteins were denatured by boiling in a water bath for 10 min. Excess OP was removed by dialysis against 10 mM ammonium bicarbonate. The 1 mg of dialyzed tubulin was digested with 0.02 mg of Promega trypsin at 37 °C for 16 h.

2.3. Purification of FP-biotinylated peptides on monomeric avidin beads

The trypsin-digested, FP-biotinylated tubulin was boiled for 10 min to denature trypsin. This prevented digestion of avidin protein by trypsin. The digest was loaded on a 1 ml column of monomeric avidin beads. The column was washed with 20 ml of 1 M Tris–Cl pH 8.5 to wash off unbound peptides, followed by 20 ml of 0.1 M Tris–Cl pH 8.5, and 20 ml of 10 mM ammonium bicarbonate. Salts were washed off with 20 ml water, before the peptides were eluted with 10 ml of 10% acetic acid. One millilitre fractions were collected.

2.4. HPLC purification

Peptides intended for infusion on the Q-Trap mass spectrometer were purified by reverse phase HPLC. The advantage of offline HPLC purification was the large amount of peptide sample that could be loaded on the C18 column (Phenomenex Prodigy 5 micron ODS size 100 × 4.60 mm). Peptides from a 1 mg tubulin digest were eluted with a gradient that started with 100% of 0.1% trifluoroacetic acid and increased to 60% acetonitrile/40% 0.1% trifluoroacetic acid in 60 min on a Waters 625 LC system. Fractions of 1 ml were collected, analyzed by MALDI-TOF mass spectrometry, and dried in a SpeedVac.

2.5. MALDI-TOF mass spectrometry

The MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems) was used for analysis of tryptic peptides prior to more rigorous analysis with the Q-Trap. This mass spectrometer was also used for analysis of tryptic peptides. A 0.5 μl sample was spotted on a 384 well Opti-TOF plate (P/N 1016491, Applied Biosystems) and the air dried spot was overlaid with 0.5 μl of 10 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% trifluoroacetic acid. Mass spectra were collected in positive ion reflector mode on a MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems, Foster City, CA). The final spectrum was the average of 500 laser shots. Masses were calibrated using CalMix 5 (Applied Biosystems).

2.6. Q-Trap 4000 mass spectrometry

Peptides from selected HPLC fractions were dissolved in 100 μl of 50% acetonitrile, 0.1% formic acid and infused into the Q-Trap 4000 linear ion trap mass spectrometer (Applied Biosystems) via a nanospray source, using a continuous flow head, a flow rate of 0.30 μl/min, and an ion spray potential of 1900 V. Spray was through a distal coated silica tip emitter FS360-75-15-D (New Objective, Woburn, MA). Mass spectra were obtained using the trap function at 4000 amu/s with dynamic fill to determine the filling time for the trap. One hundred to 350 spectra were averaged. Peptide fragmentation also employed the trap. MS/MS spectra were obtained by collision induced dissociation (CID) at a nitrogen gas pressure of 40 μTorr and a collision energy of 30–60 V. The spectrometer was calibrated on selected fragments from the MS/MS spectrum of [Glu]-fibrinopeptide B.

3. Results

3.1. Strategy for identifying labeled residues in tubulin

The strategy is to first use FP-biotin to label the tubulin. FP-biotinylated peptides are easy to find because the biotin tag gives a signature fragmentation pattern. Masses of 227, 312, and 329 amu are always present in the MS/MS scan of an FP-biotin labeled peptide [9]. We use the MS/MS function of the MALDI-TOF-TOF mass spectrometer to screen for FP-biotin labeled peptides. Then we use the Q-Trap 4000 mass spectrometer to fragment the peptides for de novo

Table 1
Theoretical masses of bovine tubulin tryptic peptides covalently labeled by OP

Tubulin chain	Sequence	Mass (*m*/*z*)	+120 sarin	+136 CPO	+162 soman	+164 DFP	+572 FP-biotin
Alpha	TGTYR	597.3	717.3	733.3	759.3	761.3	1169.3
Beta	YVPR	534.3	654.3	670.3	696.3	698.3	1106.3
Beta	GSQQYR	738.4	858.4	874.4	900.4	902.4	1310.4
Beta	EEYPDR	808.3	928.3	944.3	970.3	972.3	1380.3

Accession # gi: 73586894 for alpha-tubulin and gi: 75773583 for beta-tubulin in the NCBInr database.

sequencing to identify the site of covalent attachment of FP-biotin.

In a second phase, the protein is labeled with other OP. In the first round of screening for peptides labeled with these other OP, the assumption is made that the sites labeled by FP-biotin are also labeled by other OP. This assumption allows one to calculate theoretical OP-peptide masses and to look for the presence of these masses in the HPLC-fractionated, tryptic digest using the MALDI-TOF-TOF mass spectrometer. However, this assumption may not hold for all OP. Therefore a second strategy is used. Peptide masses observed in the MS scan for OP-labeled peptides are compared with theoretical masses for unlabeled peptides. The list of theoretical masses is generated with Protein Prospector software (UCSF). This free software is available at http://prospector.ucsf.edu. Candidates for OP-labeled peptides are chosen when their masses are equal to the sum of the known peptide mass and the added mass from the OP. These putative, OP-labeled peptides are further tested by CID fragmentation in the Q-Trap 4000 mass spectrometer, followed by manual de novo sequencing to identify the site of covalent, OP attachment.

3.2. Four tubulin peptides are labeled by OP

The structures of the OP studied in the present report are shown in Fig. 1. A portion of each OP and the phenolic proton from the labeled tyrosine are displaced when the OP makes a covalent bond with tubulin, so that the mass added to tubulin is less than the mass of the OP. The added masses are 120 amu for sarin, 136 amu for CPO, 162 amu for soman, 164 amu for DFP, and 572 amu for FP-biotin. The leaving group is fluoride ion for sarin, soman, DFP and FP-biotin, and is 3,5,6-trichloro-2-(*O*)-pyridine for CPO.

Fig. 2 shows the tubulin tryptic peptides that are targets for OP-labeling, before (panel A) and after treatment with OP (panels B–F). The peaks at 534.4, 597.4, 738.5 and 808.5 *m*/*z* in panel A are unlabeled peptides with the sequences YVPR, TGTYR, GSQQYR, and EEYPDR. After treatment of tubulin with OP, new peaks appear whose masses correspond to some of the expected, theoretical masses for OP-labeled peptides (Table 1).

Unlabeled active site peptides were present in each digest, indicating that modification by OP was incomplete. The relative amount of labeled and unlabeled peptide was calculated from isotope cluster areas. The results are summarized in Table 2. FP-biotin, DFP, and CPO reacted with all four peptides, whereas sarin and soman reacted with only one peptide. Soman and sarin concentrations were signifi-

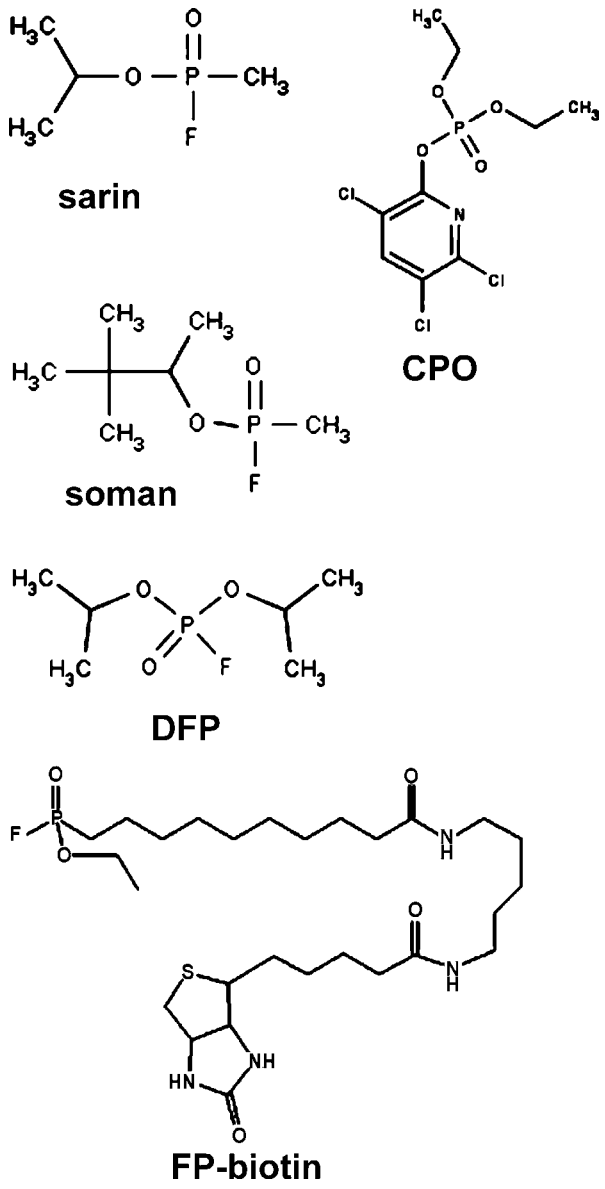

Fig. 1. OP structures.

Table 2
Percent of each peptide labeled by OP

Sequence	% labeled				
	Sarin	CPO	Soman	DFP	FP-biotin
TGTYR		43		7	70
YVPR		19	2	6	54
GSQQYR	54	23		13	66
EEYPDR		21		12	62

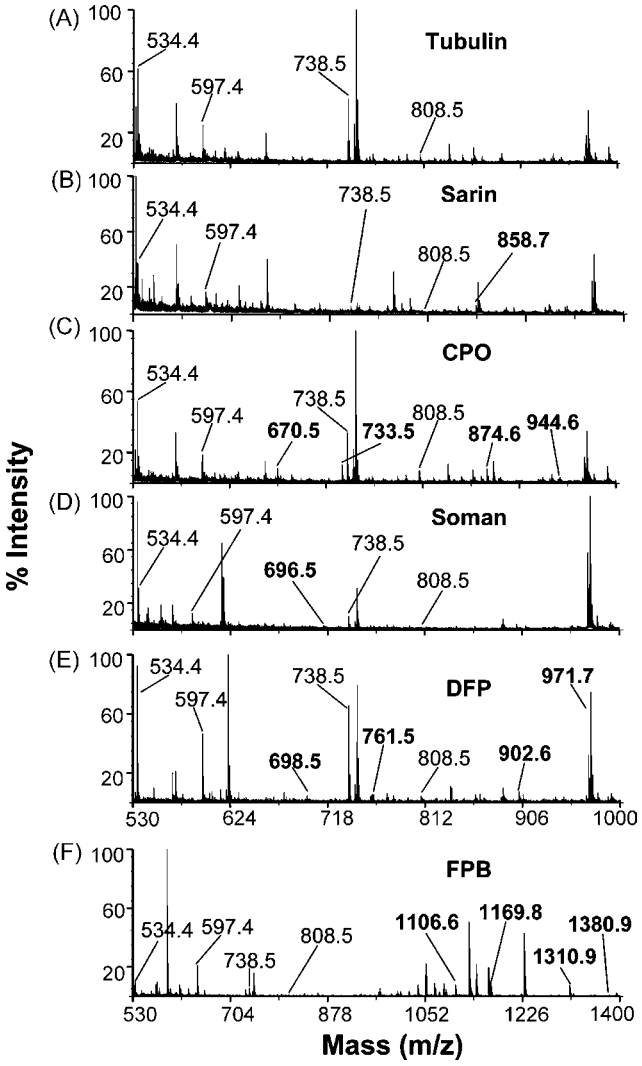

Fig. 2. Mass spectra of tryptic peptides of bovine tubulin, before and after labeling with OP. Peptides from (A) control bovine tubulin, (B) sarin treated tubulin, (C) CPO treated tubulin, E) DFP treated tubulin, (D) soman treated tubulin, (F) FP-biotin treated tubulin. OP-labeled peptide masses are in bold. One peptide was labeled with sarin and soman; four peptides were labeled with CPO, DFP, and FP-biotin.

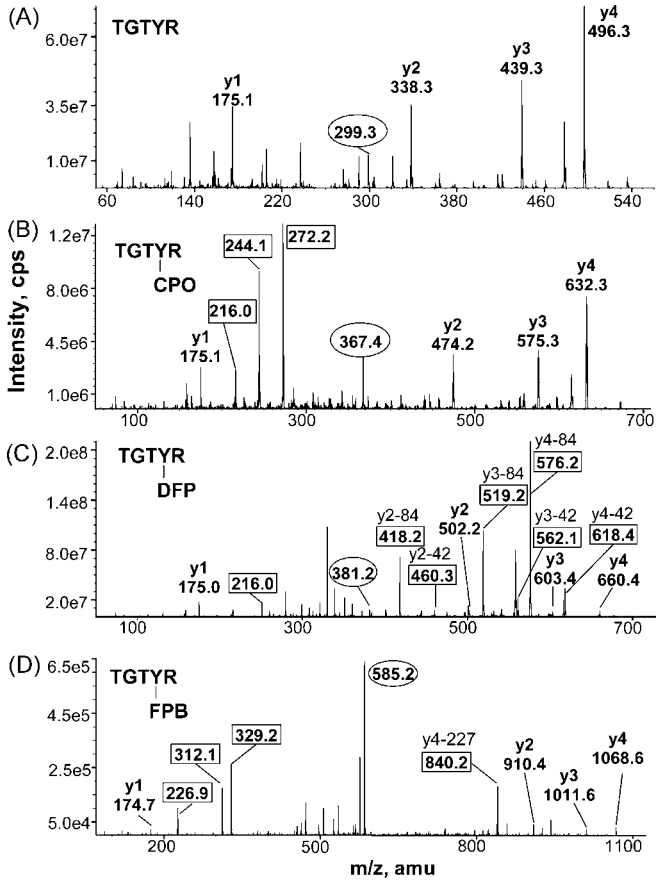

Fig. 3. MS/MS spectra of the TGTYR peptide of alpha tubulin. (A) Singly charged y ions derived from the doubly-charged, unlabeled parent ion at 299.3 *m*/*z* are shown. (B) CPO-labeled TGTYR has a doubly charged parent ion of 367.4 *m*/*z*. The y ion masses are consistent with diethylphosphate attached to tyrosine. The mass at 244 *m*/*z* is phosphotyrosine, the mass at 216 *m*/*z* is the immonium ion of phosphotyrosine, and the mass at 272 *m*/*z* is monoethylphosphotyrosine. (C) DFP-labeled TGTYR has a doubly charged parent ion of 381.2 *m*/*z*. The y ion masses are consistent with diisopropylphosphate attached to tyrosine. Masses enclosed in boxes are y ions that have lost one (42 amu) or both (84 amu) isopropyl groups. The ion at 216 *m*/*z* is the immonium ion of phosphotyrosine. (D) FP-biotin labeled TGTYR has a doubly charged parent ion of 585.2 *m*/*z*. The y ion masses are consistent with FP-biotin attached to tyrosine. The ions at 227, 312, and 329 *m*/*z* are fragments of FP-biotin. The ion at 840.2 *m*/*z* is the y4 ion that has lost 227 amu from FP-biotin.

cantly lower than the concentrations of the other OP during the labeling reaction, which might explain why fewer peptides were labeled.

3.3. Tyrosine covalently modified by OP

Collision induced fragmentation in the Q-Trap mass spectrometer conclusively identified the amino acid sequence of each labeled peptide and the residue covalently modified by OP. The MS/MS spectra in Figs. 3–6 show the y ions of each unlabeled peptide (panel A), and the same peptide after covalent modification by sarin, CPO, soman, DFP, and FP-biotin (panels B–F). The masses exactly fit the indicated sequence and fit the interpretation that the OP is attached to tyrosine (Table 3).

Ions at 214, 216, 244, and 272 *m*/*z* provide additional evidence that the OP bind to tyrosine (see the figure legends for details).

Table 3
OP-labeled tyrosines in bovine tubulin

Tubulin chain	Sequence	OP-labeled tyrosine	Location in crystal structure
Alpha	TGTYR	Tyr 83	Loop between H2 and S3
Beta	YVPR	Tyr 59	Part of the loop between H1-S2; makes lateral contact between protofilaments [10]
Beta	GSQQYR	Tyr 281	Part of the M loop between S7 and H9; makes lateral contact between protofilaments [10]
Beta	EEYPDR	Tyr 159	At the C-terminus of helix H4: residues 157–176 bind ribose of GTP [11]

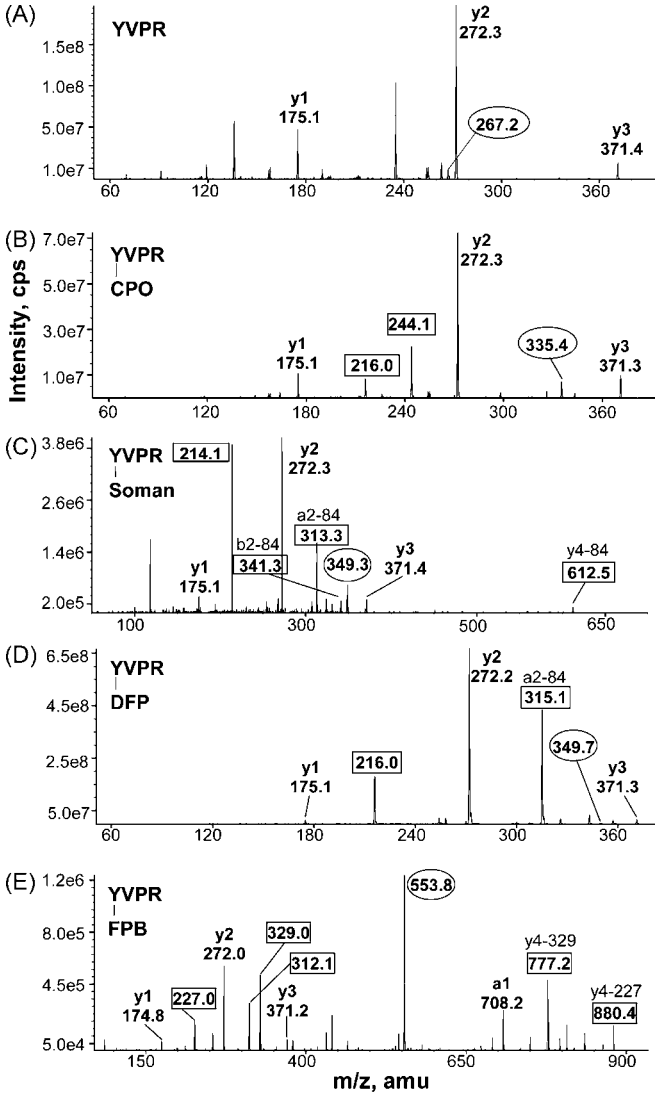

Fig. 4. MS/MS spectra of the YVPR peptide of beta tubulin. (A) Singly charged y ions derived from the doubly charged, unlabeled parent ion at 267.2 *m*/*z* are shown. (B) CPO-labeled YVPR has a doubly charged parent ion of 335.4 *m*/*z*. The y ion masses are consistent with diethylphosphate attached to tyrosine. The 216 *m*/*z* ion is the immonium ion of phosphotyrosine. The 244.1 *m*/*z* ion is phosphotyrosine. (C) Soman labeled YVPR had a doubly charged parent ion at 349 m/z. The 214 m/z ion is the immonium ion of methylphosphotyrosine. Loss of the pinacolyl group of soman reduces the mass of the singly charged parent ion by 84 amu to yield the 612 *m*/*z*, ion. Loss of the pinacolyl group also yields the b2 ion at 341 *m*/*z*, and the a2 ion at 313.3 *m*/*z*. (D) DFP-labeled YVPR has a doubly charged parent ion at 349.7 *m*/*z*. The a2 ion has lost both isopropyl groups (84 amu) to yield 315.1 *m*/*z*. (E) FP-biotin labeled YVPR has a doubly charged parent ion at 553.8 *m*/*z*. Ions at 227, 312, and 329 *m*/*z* are fragments of FP-biotin. The ions at 777.2 and 880.4 *m*/*z* are the parent ion that has lost 329 or 227 amu from FP-biotin, respectively.

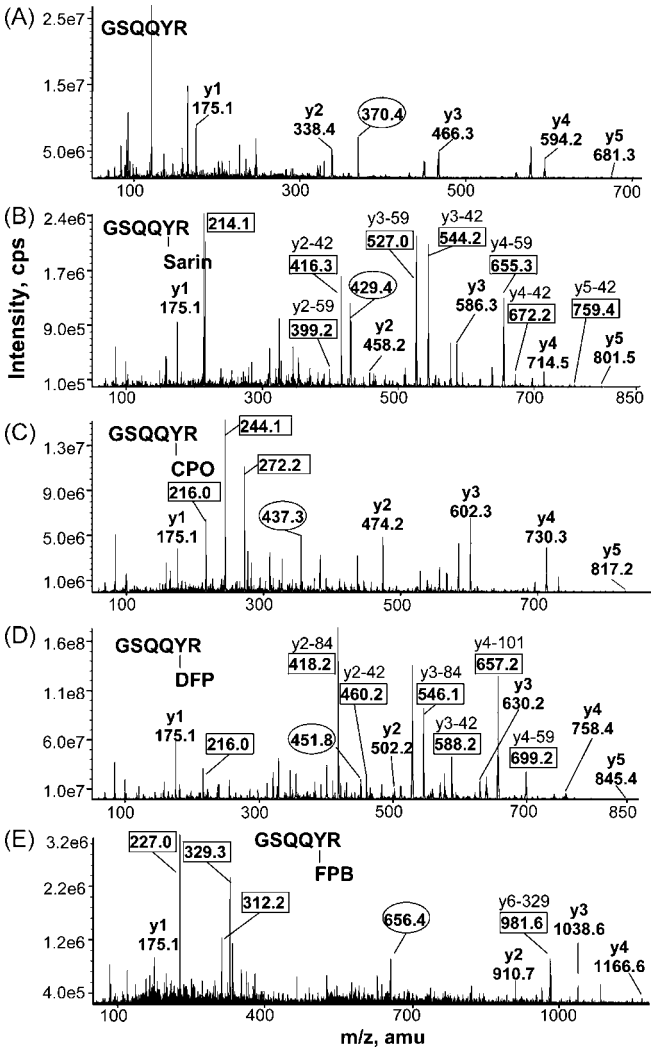

Fig. 5. MS/MS spectra of the GSQQYR peptide of beta tubulin. (A) Singly charged y ions derived from the doubly charged, unlabeled parent ion at 370.4 *m*/*z* are shown. (B) Sarin-GSQQYR had a doubly charged parent ion at 429.4 *m*/*z* and the immonium methylphosphotyrosine ion at 214 *m*/*z*. Loss of the isopropyl group reduces the y ion masses by 42 amu. Loss of the isopropyl group plus loss of an NH_3 group reduces the y ion masses by 59 amu. (C) CPO-GSQQYR had a doubly charged parent ion at 437.3 *m*/*z*. The immonium ion of phosphotyrosine is at 216 *m*/*z*. The phosphotyrosine ion is at 244.1 *m*/*z*. The monoethylphosphotyrosine ion is at 272.2 *m*/*z*. (D) DFP-GSQQYR has a doubly charged parent ion at 451.8 *m*/*z* and the immonium phosphotyrosine ion at 216 *m*/*z*. Loss of one or both isopropyl groups from diisopropylphosphate yields y ions whose masses are reduced by 42 or 84 amu. An additional loss of 17 amu yields y ions that have lost 101 or 59 amu. (E) FP-biotin-GSQQYR had a doubly charged parent ion at 656.4 *m*/*z*. Fragments derived from FP-biotin are at 227, 312, and 329 *m*/*z*. The singly charged ion at 981.6 *m*/*z* is consistent with the parent ion after loss of 329 amu from FP-biotin.

3.4. Fragmentation patterns characteristic of a particular OP

The data from Figs. 3–6 reveal CID fragmentation patterns that are characteristic of particular OP. For example, DFP-peptides readily release one or both isopropyl to yield y ions missing either 42 or 84 amu. CPO-labeled peptides yield intense peaks at 216, 244 and 272 *m*/*z* that are consistent with phosphotyrosine immonium ion, phosphotyrosine and monoethylphosphotyrosine, respectively. The FP-biotinylated peptides release the characteristic fragments of FP-biotin at 227, 312, and 329 *m*/*z*. Singly charged ions missing either 227 or 329 amu were common. The pinacolyl group of soman was released from soman labeled YVPR peptide (Fig. 4C) to yield ions missing 84 amu. The isopropyl group of sarin was released from sarin labeled GSQQYR peptide (Fig. 5B) to yield ions missing 42 amu. Peptides that had lost 42 amu fragmented further to release NH_3 (17 amu), yielding an ion pair characteristic of sarin labeling.

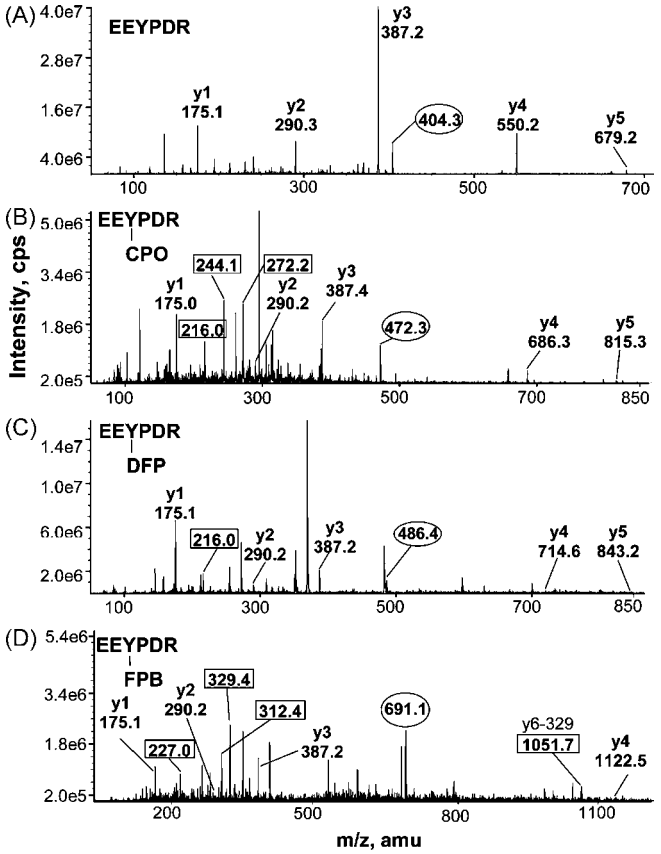

Fig. 6. MS/MS spectra of the EEYPDR peptide of beta tubulin. (A) Singly charged y ions derived from the doubly charged, unlabeled parent at 404.3 *m*/*z* are shown. (B) CPO-EEYPDR has a doubly charged parent ion at 472.3 *m*/*z*. The immonium phosphotyrosine ion is at 216 *m*/*z*. Phosphotyrosine is at 244 *m*/*z* and monoethylphosphotyrosine is at 272 *m*/*z*. (C) DFP-EEYPDR has a doubly charged parent ion at 486.4 *m*/*z* and an immonium phosphotyrosine ion at 216 *m*/*z*. (D) FP-biotin EEYPDR has a doubly charged parent ion at 691.1 *m*/*z*. Fragment ions of FP-biotin are present at 227, 312, and 329 *m*/*z*. The singly charged parent ion that has lost 329 amu from FP-biotin is at 1051.7 *m*/*z*.

In no case was the entire OP released from tyrosine. The phosphate group remained bound to tyrosine during CID fragmentation. This contrasts with OP bound to serine where the fragmentation process releases the entire bound OP, leaving no trace of the OP behind, and yielding dehydroAlanine in place of the OP-labeled serine [12].

3.5. No aging

When soman, sarin, or DFP are bound to acetylcholinesterase or butyrylcholinesterase they rapidly lose an alkyl group in a process called aging [13–15]. An aged soman labeled peptide would have an added mass of 78 amu rather than 162; an aged sarin labeled peptide would have an added mass of 78 amu rather than 120; an aged DFP labeled peptide would have an added mass of 122 amu rather than 164 in the MS spectrum. No evidence of aging was found in OP labeled tubulin peptides as no masses representing aged OP-peptides were found in MS scans. We conclude that tubulin OP adducts on tyrosine do not age.

4. Discussion

4.1. Tyrosine as a motif for OP labeling

The literature overwhelmingly supports the fact that OP bind covalently to an active site serine within the consensus sequence GXSXG. Enzymes are defined as serine hydrolases when their activity is inhibited by OP. On the other hand, covalent binding of OP to tyrosine has previously been reported only for albumin [16–19], papain [20] and bromelain [21]. Our previous mass spectrometry work identified the OP binding site in human albumin as Tyr 411, and in bovine albumin as Tyr 410 [9,16]. The present report of OP binding to tyrosine in alpha and beta tubulin is novel. No covalent attachment site for OP binding to tubulin has previously been reported.

No obvious consensus binding site can be deduced from the four peptides reported here, though each target peptide contains a positively charged arginine within three residues of the labeled tyrosine which may serve to reduce the pK_a of the tyrosine hydroxyl group, and thereby activate it.

4.2. Significance of OP-labeling of tubulin

The crystal structure of tubulin shows that each of the OP-labeled tyrosines is located in a region where protofilaments interact laterally, or bind GTP [10,11]. OP-binding to tubulin could therefore alter tubulin conformation or GTP binding. A change in tubulin conformation or GTP binding could explain the observation of Prendergast et al. that CPO inhibits tubulin polymerization [8]. When tubulin does not polymerize, microtubules do not form, and nutrient transportation from the nerve cell body to the nerve synapse is disrupted. It is concluded that OP bind covalently to tubulin, and that this binding could explain the axonal transport deficits and cognitive impairment previously associated with OP exposure [5,6].

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Pseudo-esterase Activity of Human Albumin

SLOW TURNOVER ON TYROSINE 411 AND STABLE ACETYLATION OF 82 RESIDUES INCLUDING 59 LYSINES[*] [S]

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Human albumin is thought to hydrolyze esters because multiple equivalents of product are formed for each equivalent of albumin. Esterase activity with *p*-nitrophenyl acetate has been attributed to turnover at tyrosine 411. However, *p*-nitrophenyl acetate creates multiple, stable, acetylated adducts, a property contrary to turnover. Our goal was to identify residues that become acetylated by *p*-nitrophenyl acetate and determine the relationship between stable adduct formation and turnover. Fatty acid-free human albumin was treated with 0.5 mM *p*-nitrophenyl acetate for 5 min to 2 weeks, or with 10 mM *p*-nitrophenyl acetate for 48 h to 2 weeks. Aliquots were digested with pepsin, trypsin, or GluC and analyzed by mass spectrometry to identify labeled residues. Only Tyr-411 was acetylated within the first 5 min of reaction with 0.5 mM *p*-nitrophenyl acetate. After 0.5–6 h there was partial acetylation of 16–17 residues including Asp-1, Lys-4, Lys-12, Tyr-411, Lys-413, and Lys-414. Treatment with 10 mM *p*-nitrophenyl acetate resulted in acetylation of 59 lysines, 10 serines, 8 threonines, 4 tyrosines, and Asp-1. When Tyr-411 was blocked with diisopropylfluorophosphate or chlorpyrifos oxon, albumin had normal esterase activity with β-naphthyl acetate as visualized on a nondenaturing gel. However, after 82 residues had been acetylated, esterase activity was almost completely inhibited. The half-life for deacetylation of Tyr-411 at pH 8.0, 22 °C was 61 ± 4 h. Acetylated lysines formed adducts that were even more stable. In conclusion, the pseudo-esterase activity of albumin is the result of irreversible acetylation of 82 residues and is not the result of turnover.

Human albumin has been reported to have esterase activity with *p*-nitrophenyl acetate (1, 2), α-naphthyl acetate, phenyl acetate, 1-naphthyl *N*-methylcarbamate (3), β-naphthyl acetate (4), aspirin (5), ketoprofen glucuronide (6), carprofen acylglucuronide (7), cyclophosphamide (8), nicotinate esters (9), long- and short-chain fatty acid esters (10), octanoyl ghrelin (11), organophosphorus pesticides (12), carbaryl (13), *o*-nitrotrifluoroacetanilide (14), *o*-nitroacetanilide (15), and nerve agents (16).

One site in albumin is rapidly acetylated by *p*-nitrophenyl acetate, showing a burst of product, but up to 5.2 molar equivalents are incorporated when albumin is treated with a 9-fold excess of *p*-nitrophenyl [^{14}C]acetate (1). The 5 equivalents of label are not removable by extensive dialysis. The esteratic site has been identified as Tyr-411 based on site-directed mutagenesis studies (17). Mass spectrometry (MS)[2] has identified Tyr-411 as the residue labeled by organophosphorus esters including diisopropylfluorophosphate (DFP), soman, sarin, dichlorvos, FP-biotin, and chlorpyrifos oxon (16, 18) confirming the report by Sanger that a tyrosine in albumin is labeled by DFP (19),and reports that a tyrosine in albumin is labeled by the nerve agents soman, sarin, cyclosarin, and tabun (20). When albumin is labeled with 1 mol of DFP, albumin loses the fast phase of its esterase activity (the burst), supporting the conclusion that DFP and *p*-nitrophenyl acetate bind to the same site, namely to Tyr-411 (21).

Our goal was to identify the additional sites labeled by *p*-nitrophenyl acetate. We wanted an explanation for the apparently inconsistent observation that albumin hydrolyzes *p*-nitrophenyl acetate at measurable rates, yet the ^{14}C-acetylated albumin formed by *p*-nitrophenyl acetate is a stable adduct.

EXPERIMENTAL PROCEDURES

Materials—A 1 mg/ml solution of fatty acid-free human albumin (Fluka 05418, via Sigma) was dissolved in 10 mM Tris-Cl, pH 8.0. A 1 mg/ml solution of porcine pepsin (Sigma P6887) in 10 mM HCl was stored at −80 °C. Sequencing grade modified trypsin (Promega V5113) at a concentration of 20 μg in 50 μl of 50 mM acetic acid was stored at −80 °C. Endoproteinase GluC (*Staphylococcus aureus* protease V8) was from Worthington Biochemical Corp (Lakewood, NJ, LS003608). 1 mg of GluC powder was dissolved in 210 μl of water, aliquoted into micro-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.

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[2] The abbreviations used are: MS, mass spectrometry; DFP, diisopropylfluorophosphate; amu, atomic mass unit; CHCA, α-cyano-4-hydroxycinnamic acid; LC/MS/MS, liquid chromatography tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time of flight mass spectrometry; i.d., inner diameter.

centrifuge tubes each containing 10 μl (50 μg), dried in a vacuum centrifuge, and stored at −80 °C. A 10 mg/ml solution of α-cyano-4-hydroxycinnamic acid matrix (CHCA) (Applied Biosystems) in 50% acetonitrile, 0.1% trifluoroacetic acid was stored at room temperature. A 0.1 M solution of *p*-nitrophenyl acetate (Sigma N8130) in acetonitrile was stored at −20 °C. DFP, a liquid with a concentration of 5.73 M, was from Sigma (D0879). Chlorpyrifos oxon (ChemService Inc. West Chester, PA; MET-674B) was dissolved in methanol to make a 1 M solution and stored at −80 °C. β-naphthyl acetate, Fast Blue RR, and fluorodinitrobenzene (>99% pure) were from Sigma. Albumin samples were concentrated in a Microcon centrifugal device with a YM10 membrane whose molecular weight cutoff was 10,000 (Millipore 42406).

Pure Albumin Labeled with p-Nitrophenyl Acetate, Digested with Pepsin While Albumin Disulfides Were Intact—1 ml of a 1 mg/ml solution of fatty acid-free human albumin (15 μM) in 10 mM Tris-Cl, pH 8.0 was treated with a 33-fold molar excess of *p*-nitrophenyl acetate (5 μl of 0.1 M in acetonitrile) at 22 °C. 1 ml of control albumin solution was treated with 5 μl of acetonitrile. A 50-μl aliquot was removed into 50 μl of 1% trifluoroacetic acid containing 2 μl of 1 mg/ml pepsin after 5, 10, 15, 20, 30, 40, 50, 60 min, 6 h, 24 h, 9 days, and 14 days. The drop in pH to 1.4 stopped the reaction with *p*-nitrophenyl acetate. Digestion with pepsin was at 37 °C for 1 to 2 h. A 0.5-μl aliquot of the digest was spotted on a MALDI target plate, dried, and overlaid with 0.5 μl of CHCA matrix, for analysis in a MALDI-TOF/TOF 4800 mass spectrometer.

Absorbance at 400 nm to Measure p-Nitrophenolate Produced by Albumin Hydrolysis of p-Nitrophenyl Acetate—1 ml of 1 mg/ml human albumin in 10 mM Tris-Cl, pH 8.0 was incubated with 5 μl of 0.1 M *p*-nitrophenyl acetate at 22 °C. At various times after mixing, a 50-μl aliquot was withdrawn and diluted to 2 ml for measurement of absorbance at 400 nm. The reaction was followed until all of the *p*-nitrophenyl acetate was consumed. A control sample, without albumin, was followed in a similar manner to measure spontaneous hydrolysis of *p*-nitrophenyl acetate, in 10 mM Tris-Cl, pH 8.0. The amount of *p*-nitrophenylate ion formed in the albumin reaction was corrected for spontaneous hydrolysis.

The extinction coefficient for the *p*-nitrophenolate ion at pH 8.0 is 16,900 M^{-1} cm^{-1} at 400 nm (1). Therefore, the completely hydrolyzed 500 μM *p*-nitrophenyl acetate should have an absorbance at 400 nm of 8.45.

Albumin Incubated with Potassium Acetate—To determine whether acetylation of lysines was accomplished by reaction with *p*-nitrophenyl acetate or by reaction with free acetate, we measured acetylated lysines after incubation of albumin with potassium acetate. 1 ml of 1 mg/ml albumin in 10 mM Tris-Cl, pH 8.0 was incubated with 500 μM potassium acetate for 15 h at 22 °C. A 50-μl aliquot of the reaction mixture was removed into 50 μl of 1% trifluoroacetic acid containing 2 μl of 1 mg/ml pepsin and incubated for 2 h at 37 °C. The digest was analyzed with a MALDI-TOF-TOF 4800 mass spectrometer, with CHCA matrix.

HPLC Purification of the 1872-amu Acetylated Peptide LVRYTKKVPQVSTPTL—This peptide was purified to make it possible to get an MS/MS spectrum of acetylated YTK. The acetylated YTK peptide ionized in the mass spectrometer only when the number of ions in the mixture was minimal. 10 mg of human albumin in 1 ml of 10 mM Tris-Cl, pH 8.0 was treated with a 33-fold molar excess of *p*-nitrophenyl acetate for 5 min. The reaction was stopped by the addition of 1 ml of 1% trifluoroacetic acid and 0.1 ml of 1 mg/ml pepsin. After 1 h at 37 °C, the pepsin-digested albumin was purified on a Waters 625 LC system on a Phenomenex Prodigy 5 micron C18 100 × 4.60 mm column. Peptides were eluted with a 60-min gradient from 0.1% trifluoroacetic acid in water to 60% acetonitrile, 0.09% trifluoroacetic acid at a flow rate of 1 ml/min. An aliquot from each 1-min fraction was spotted on a MALDI target plate to identify the fractions that contained the 1872 mass. It was found that the 1872 mass eluted with 26% acetonitrile in fractions 26 and 27. These fractions were combined, dried, and injected into the same Phenomenex column, but this time the elution solvents were 10 mM potassium phosphate pH 7.0 and acetonitrile. In a 60-min gradient from 0 to 60% acetonitrile, the 1872 mass eluted in fractions 37–44. About 5000 pmol of the purified 1872-amu peptide in 225 μl of 50 mM ammonium bicarbonate were digested with 2 μg of Promega trypsin for 4 h at 37 °C, dried, dissolved in 50 μl of 5% acetonitrile, 0.1% formic acid, and infused into the QTRAP 4000 mass spectrometer.

Percent Acetylation Calculated from Cluster Areas in a MALDI-TOF-TOF 4800 (Applied Biosystems, Foster City) Mass Spectrometer—Essentially salt-free 0.5-μl samples were spotted on a MALDI target plate, air-dried, and overlaid with 0.5 μl of 10 mg/ml CHCA in 50% acetonitrile, 0.1% trifluoroacetic acid. MS spectra were acquired with laser power at 3000 volts in positive reflector mode. Each spectrum was the average of 500 laser shots. The percentage of acetylation was calculated by dividing the cluster area of the unlabeled peptide by the sum of the cluster areas for the unlabeled and labeled peaks. The mass spectrometer was calibrated against des-Arg-bradykinin (904.468 Da), angiotensin 1 (1296.685 Da), Glu-fibrinopeptide B (1570.677 Da), and neurotensin (1672.918 Da) (Cal Mix 1 from Applied Biosystems).

LC/MS/MS with the QTRAP 2000 and LCQ Deca XP Mass Spectrometers—Human albumin treated with *p*-nitrophenyl acetate was denatured by boiling 10 min in the presence of 10 mM dithiothreitol, carbamidomethylated with 90 mM iodoacetamide, and dialyzed against 2 × 4 liters of 10 mM ammonium bicarbonate. A 500-μg aliquot was digested with 10 μg of Promega trypsin overnight at 37 °C, or with 50 μg of GluC. The digest was dried in a vacuum centrifuge and dissolved in 5% acetonitrile, 0.1% formic acid to make 6.7 pmol/μl. A 10-μl aliquot was injected into the HPLC nanocolumn (218MS3.07515 Vydac C18 polymeric rev-phase, 75 μm i.d. × 150 mm long; P.J. Cobert Assoc, St. Louis, MO). Peptides were separated with a 90-min linear gradient from 0 to 60% acetonitrile at a flow rate of 0.3 μl/min and electrosprayed through a fused silica emitter (360 μm o.d., 75 μm i.d., 15 μm taper, New Objective) directly into the QTRAP 2000 (Applied Biosystems, Foster City, CA), a hybrid quadrupole linear ion trap mass spectrometer. An ion-spray voltage of 1900 V was maintained between the emitter and the mass spectrometer. Information-dependent acquisition was used to collect MS, enhanced MS, and MS/MS spectra for the three most intense peaks in each

cycle, having a charge of +1 to +4, a mass between 200 and 1700 *m/z*, and an intensity >10,000 cps. All spectra were collected in the enhanced mode, using the trap function. Precursor ions were excluded for 30 s after one MS/MS spectrum had been collected. The collision cell was pressurized to 40 μTorr with pure nitrogen and collision energies between 20 and 40 eV were determined automatically by the software based on the mass and charge of the precursor ion. The mass spectrometer was calibrated on selected fragments from the MS/MS spectrum of Glu-fibrinopeptide B. The MS/MS data were processed using Analyst 1.4.1 software and submitted to Mascot for identification of peptide sequences (22).

A pepsin digest of the same carbamidomethylated preparation was analyzed on the LCQ Deca XP mass spectrometer (Thermo-Finnigan, San Jose, CA) coupled to the Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA). The 75-μm i.d. C18 column was from LCPackings. HPLC solvents were: A) 0.1 M acetic acid and B) 80% acetonitrile, 0.1 M acetic acid. Peptides were eluted with a gradient from 0 to 55% B in 90 min. Data were collected with Xcalibur 2.0 and analyzed with OMMSA search engine (23).

Infusion in QTRAP 4000 Mass Spectrometer—Peptides dissolved in 50% acetonitrile, 0.1% formic acid were infused into the QTRAP 4000 (Applied Biosystems, Foster City, CA) mass spectrometer at a flow rate of 0.3 μl/min through an 8-μm emitter (FS360-50-8-D, New Objective) via a 25-μl Hamilton syringe mounted on a Harvard syringe pump. 500 MS/MS spectra were accumulated for each parent ion.

Deacetylation of Tyr-411—The production and disappearance of acetylated Tyr-411 was followed by MALDI-TOF mass spectrometry. A 15 μM solution of human albumin in 10 mM Tris-Cl, pH 8.0 was treated with an equimolar concentration of *p*-nitrophenyl acetate at 22 °C. After various times, a 10-μl aliquot was mixed with 10 μl of 1% trifluoroacetic acid and 1 μl of 1 mg/ml pepsin. After 1–2 h at 37 °C a 0.5 μl aliquot of the digest was spotted on a MALDI target plate. Cluster areas for the unlabeled peptides at 1717 and 1830 amu and the labeled peptides at 1759 and 1872 amu were used to calculate % labeling on Tyr-411.

Percentage of Acetylated Lysines Calculated from Amino Acid Composition Analysis—Quantitation of the acetylated lysine was obtained by the use of fluorodinitrobenzene (Allfrey *et al.*, Ref. 30). Fluorodinitrobenzene reacts with un-acetylated lysine to form ε-*N*-dinitrobenzylated lysine. Acid hydrolysis, in preparation for amino acid composition analysis hydrolyzes the ε-*N*-acetyl lysines to yield free lysine, but the dinitrobenzylated lysines are relatively stable to acid hydrolysis. The amount of free lysine appearing in the amino acid composition analysis, corrected for hydrolysis of dinitrobenzylated lysine, represents the amount of lysine that was originally acetylated.

Fluorodinitrobenzylation employs the method originally described by Sanger (24). Acetylated and control albumin were dialyzed into $NaHCO_3$ (20 mg/ml). Two hundred micrograms of dialyzed albumin were adjusted to 1 ml with $NaHCO_3$ and mixed with 2 ml of absolute ethanol. The mixture was shaken at room temperature for 1 h, at which time 30 μl of fluorodinitrobenzene was added and that mixture shaken for an additional 2 h. The insoluble yellow product was washed twice with water, twice with absolute ethanol, twice with diethyl ether, air-dried, and submitted for amino acid composition analysis.

Nondenaturing Gel Electrophoresis—Albumin esterase activity was demonstrated on a nondenaturing 4–30% polyacrylamide gradient gel, stained for esterase activity with β-naphthyl acetate and Fast Blue RR (25). Gels were shaken in 100 ml of 0.05 M Tris-Cl, pH 7.4 containing 50 mg of β-naphthylacetate in 1 ml ethanol, and 50 mg of solid Fast Blue RR. Though most of the Fast Blue RR does not dissolve, pink bands of esterase activity appear within 15–30 min. The gel was counterstained with Coomassie Blue to show protein concentration.

The positive control samples, not acetylated on any residues, were 5 μl of human serum and 200 μg of fatty acid-free human albumin. The negative control was albumin that had been denatured in 8 M urea, reduced with 10 mM dithiothreitol, carbamidomethylated with iodoacetamide, dialyzed to remove salts, and concentrated to 10 μg/μl. Albumin acetylated on 100% of Tyr-411 only, was prepared by incubating 10 mg/ml albumin with 5 mM *p*-nitrophenyl acetate (33-fold excess) in 10 mM Tris-Cl, pH 8.0 for 5 min, followed immediately by gel electrophoresis. Albumin acetylated on 100% Tyr-411 and 20% of the lysines was prepared by incubating 1 mg/ml albumin with 0.5 mM *p*-nitrophenyl acetate for 6 h at 22 °C. Albumin with free Tyr-411 and 20% acetylated lysines was prepared by incubating 1 mg/ml albumin with 0.5 mM *p*-nitrophenyl acetate, 0.01% sodium azide for 2 weeks at 22 °C, during which time Tyr-411 was completely deacetylated as shown by mass spectrometry. Albumin with 100% acetylated Tyr-411 and 60–100% acetylated lysines was prepared by incubating 1 mg/ml albumin with 10 mM *p*-nitrophenyl acetate for 48 h. Percent acetylation of Tyr-411 and lysines was determined by mass spectrometry as described above, and by amino acid composition analysis after reaction with fluorodinitrobenzene.

Albumin labeled with DFP on 80% of Tyr-411 was prepared by treating 2 mg/ml albumin with a 20-fold excess of DFP in 10 mM Tris-Cl, pH 8.0 for 2 h at 22 °C. Albumin labeled with chlorpyrifos oxon on 95% of Tyr-411 was prepared by treating 1 mg/ml albumin in 10 mM Tris-Cl, pH 8.0 with a 7.5-fold excess of chlorpyrifos oxon for 48 h at 22 °C. The percent free and modified Tyr-411 was determined by mass spectrometry. No lysines were labeled by DFP or chlorpyrifos oxon.

RESULTS

Tyrosine 411 Is Rapidly Acetylated by p-Nitrophenyl Acetate—A 5-min reaction of albumin (15 μM) with *p*-nitrophenyl acetate (500 μM) results in complete acetylation of Tyr-411. This is indicated by the mass shift of +42 amu for peptic peptides VRYTKKVPQVSTPTL and LVRYTKKVPQVSTPTL (missed cleavage), which have masses of 1717 and 1830 amu in the control albumin digest (Fig. 1*A*), but masses of 1759 and 1872 amu after treatment with *p*-nitrophenyl acetate (Fig. 1*B*). No unlabeled masses at 1717 and 1830 amu remain after treatment with *p*-nitrophenyl acetate, indicating 100% labeling. No other peptides were found to have a mass shift at this time point.

The labeled residue was identified as tyrosine 411 by fragmentation of the doubly charged parent ion 936.5 *m/z* (corresponding to the singly charged 1872 amu ion) in the QTRAP mass spectrometer. Fig. 2*A* shows fragmentation of parent ion

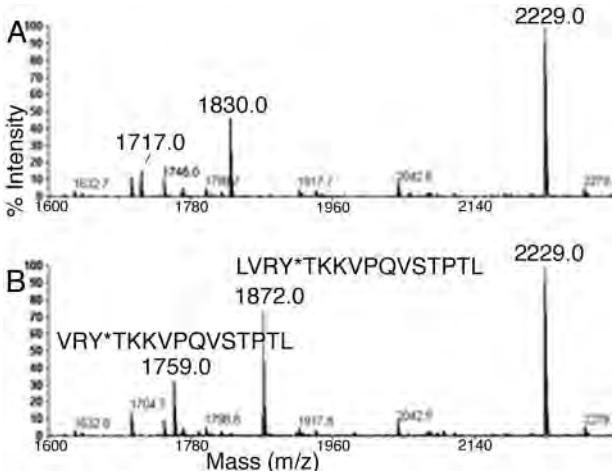

FIGURE 1. **MALDI-TOF mass spectra of peptic peptides of human albumin before (*A*) and after (*B*) 5 min reaction with 0.5 mM *p*-nitrophenyl acetate.** The mass of peptides at 1717 and 1830 amu increased by +42 amu due to acetylation of Tyr-411.

936.5 *m*/*z*. All major peaks are assigned to fragments of LVRYTKKVPQVSTPTL. The presence of the b4 ion (representing residues LVRY* plus acetyl) at 574.3 amu indicates acetylation of that fragment. The most likely candidate for acetylation is tyrosine. The b4–17 ion at 557.3 amu, and the b-ion fragments b5 ion at 675.3 amu through b13–18 at 1524.8 amu are all 42 amu larger than predicted from the amino acid sequence, further supporting acetylation of tyrosine in LVRYTKKVPQVSTPTL.

To obtain additional support for acetylation on tyrosine, peptide LVRYTKKVPQVSTPTL from Fig. 2*A* was purified by HPLC, digested with trypsin, and analyzed by mass spectrometry. Fig. 2*B* shows fragmentation of the 453.2 amu singly charged parent ion. The labeled peaks in the spectrum support the sequence YTK where the acetyl group is on the phenolic oxygen of Tyr-411. The mass at 178.1 is the *O*-acetyl-tyrosine immonium ion. The 178.1 mass was found in other peptides that had an acetylated tyrosine. A peak at 178.1 can be used as a marker for *O*-acetylated tyrosine.

There is no possibility that the acetyl group is on the amino group of tyrosine, because the reaction with *p*-nitrophenyl acetate was performed while the amino group of Tyr-411 was in a peptide bond and therefore unavailable for modification. Taken together, the data indicate that Tyr-411 is the first albumin residue to be acetylated by *p*-nitrophenyl acetate.

Lysine 413 and Lysine 414 Are Slowly Acetylated by p-Nitrophenyl Acetate—After 6 h of reaction of 15 μM albumin with 500 μM *p*-nitrophenyl acetate, a second and third site on peptides VRYTKKVPQVSTPTL and LVRYTKKVPQVSTPTL were acetylated, as indicated by peaks with mass shifts of +42, +84, and +126 amu. The 1717 amu peak (supplemental Fig. S1*A*) shifted to 1759 amu, 1801 amu, and 1843 amu (supplemental Fig. S1*B*), while the 1830 amu peak shifted to 1872, 1914, and 1956 amu (supplemental Fig. S1*B*). At 6 h, about 50% of peptides VRYTKKVPQVSTPTL and LVRYTKKVPQVSTPTL were labeled with one acetate, about 50% with 2 acetates, and 1–2% with 3 acetates (percentage estimates are based on the cluster areas of the peaks). The percentage of acetylated peptides did not increase after 6 h, because 90% of the *p*-nitrophenyl acetate had been hydrolyzed by that time. The possibility that acetate (rather than *p*-nitrophenyl acetate) might be acetylating lysines was ruled out by the finding that no mass shifts occurred when 1 mg/ml albumin was treated with 0.5 mM potassium acetate.

Peptides VRYTKKVPQVSTPTL and LVRYTKKVPQVSTPTL with a mass shift of +84 amu were acetylated on Tyr-411 and Lys-413, or on Tyr-411 and Lys-414. Supplemental Fig. S2 shows the MS/MS spectrum for doubly charged parent ion 957.5 of peptide LVRYTKKVPQVSTPTL at the 6-h time point. The b4 ion at 574.1 amu (representing residues LVRY* plus 1 acetyl) and the b5 ion at 675.7 amu (representing residues LVRY*T plus 1 acetyl) support acetylation of Tyr-411. The b6 ion at 844.3 amu (representing residues LVRY*TK* plus 2 acetyls) supports acetylation of Lys-413 in addition to Tyr-411. The existence of the b6 ion mass at 803.3 amu (representing residues LVRY*TK plus 1 acetyl) suggests that Lys-413 can appear without being acetylated. The appearance of an un-acetylated form of Lys-413 together with acetylated b7 to b11 ions is consistent with the second acetyl group on Lys-414. Other +84 ions were found (data not shown) where the second acetyl group was exclusively on Lys-413 or on Lys-414. Based on the relative intensities of the peaks at 803.3 and 844.3 amu, acetylated Lys-414 was more abundant than acetylated Lys-413. From the above analysis, it follows that triply acetylated peptides, with a mass shift of +126 amu, were acetylated on Tyr-411, Lys-413, and Lys-414.

The N Terminus of Albumin, Lysine 4, Lysine 12, and Serine 5 Are Slowly Acetylated by p-Nitrophenyl Acetate—Another prominent peptide in pepsin-digested albumin was DAHKSEVAHRFKDLGEENF at 2229 amu (supplemental Fig. S1*A*). After 6 h of reaction of 15 μM albumin with 500 μM *p*-nitrophenyl acetate, 20% of this peak had acquired a mass of +42 amu (supplemental Fig. S1*B*), 2% had acquired a mass of +84 amu, and about 0.5% had increased in mass by +126 amu. Incubation with 10 mM *p*-nitrophenyl acetate for 48 h resulted in complete disappearance of the 2229 and 2271 amu peaks and appearance of +84, +126, and +168 amu ions at 2313, 2355, and 2397 amu (data not shown). MS/MS spectra showed that the +42 amu parent ion was acetylated on Asp-1, the N terminus of albumin, the +84 amu parent ion was acetylated on Asp-1 and Lys-12, and the +126 amu ion was acetylated on Asp-1, Lys-12, and Lys-4 (data not shown).

The +168 amu ion was acetylated on Asp-1, Lys-4, Ser-5, and Lys-12 (supplemental Fig. S3). Most of the major peaks in the spectrum could be assigned to the DAHKSEVAHRFKDLGEENF peptide. The 366.1 amu mass was consistent with the b3 ion (representing D*AH plus 1 acetyl). Though histidine is a potential candidate for acetylation, *N*-acetylhistidine is unstable (26). Therefore, acetylation is on the N terminus. The mass at 536.2 amu is consistent with the b4 ion (representing D*AHK* plus 2 acetyls). The 665.2 amu mass is consistent with the b5 ion (representing D*AHK*S* plus three acetyls). Additional b-ions at 794.4 amu (b6), 893.0 amu (b7), and 964.3 amu (b8) support the presence of 3 acetyls. The mass at 1296.5 amu is consistent with the y10 ion (representing RFK*DLGEENF plus 1 acetyl) where acetylation is on the lysine.

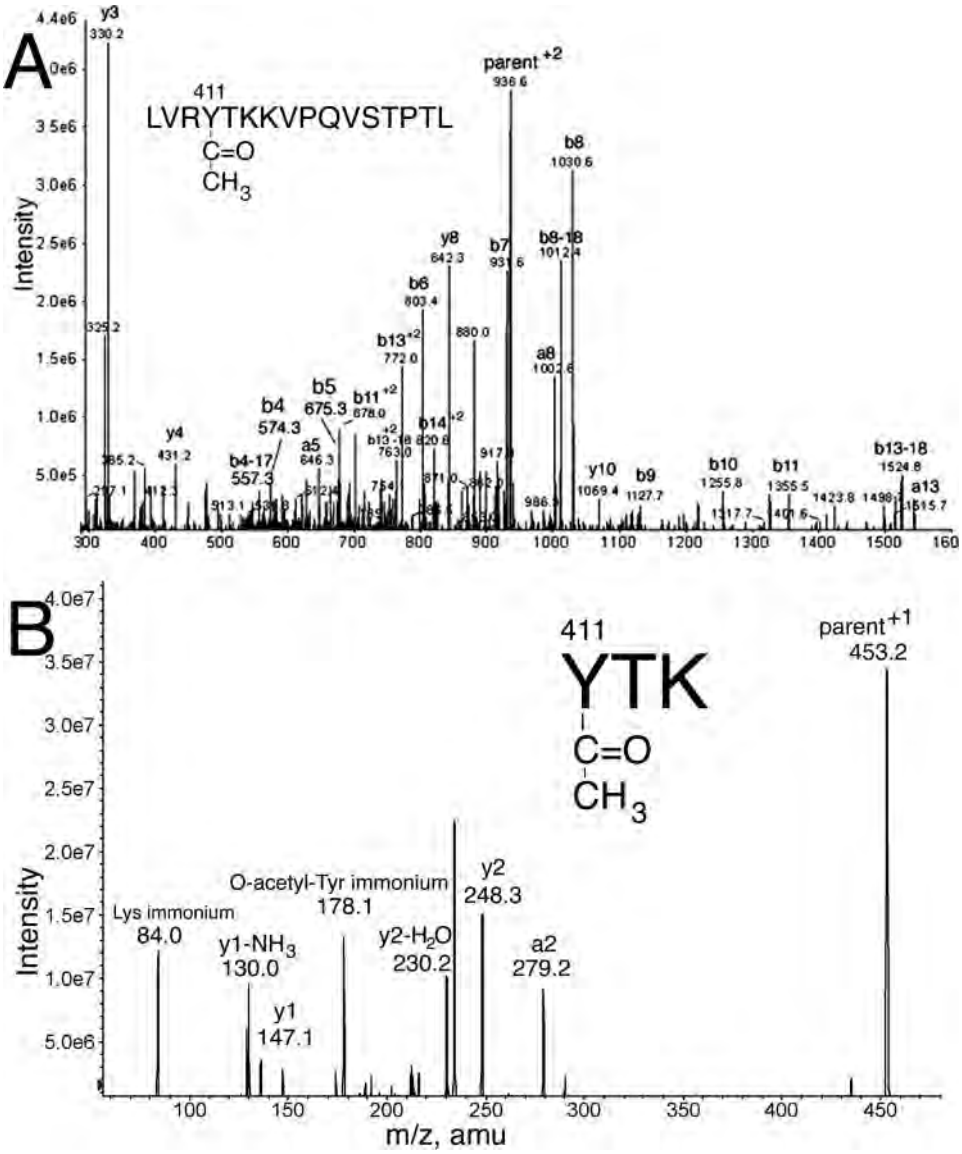

FIGURE 2. **MS/MS spectra to identify the residue acetylated after 5 min reaction of 1 mg/ml albumin with 0.5 mM p-nitrophenyl acetate.** *A*, fragmentation of the doubly charged form of peptide 1872 identifies acetylated Tyr 411 in LVRY*TKKVPQVSTPTL. *B*, fragmentation of the singly charged peptide of mass 453.2 identifies acetylated Tyr-411 in Y*TK. The peak at 178.1 is the *O*-acetyl-tyrosine immonium ion.

Analysis of cluster areas in the MALDI-TOF spectra showed that the N-terminal Asp-1 and Lys-12 were 100% acetylated, consistent with the disappearance of peaks at 2229 and 2271 amu. About 65% of Lys-4 and 6% of Ser-5 residues were acetylated, indicating that serine is not nearly as reactive with *p*-nitrophenyl acetate as lysine.

Residues Acetylated by 0.5 mM p-Nitrophenyl Acetate—Within the first 5 min of reaction of 15 μM albumin with 0.5 mM *p*-nitrophenyl acetate, Tyr-411 was acetylated 99–100%. No other residue was significantly acetylated within 5 min. Six hours after addition of *p*-nitrophenyl acetate, residues Asp-1, Lys-12, Lys-413, and Lys-414 were acetylated about 20–25% while Tyr-411 was still acetylated 100%. An additional 19 peptides had a mass shift of +42 amu as observed by MALDI-TOF, however peak intensities were low, and the peptide sequences could not be determined. After 50 h, all of the *p*-nitrophenol had been exhausted, and half of the Tyr-411 was free. By 2 weeks, none of the Tyr-411 was labeled. The albumin still carried a lot of label, but the label was on lysines and on the N-terminal Asp-1.

Residues Acetylated by 10 mM p-Nitrophenyl Acetate—Supplemental Table S1 lists the residues in human albumin acetylated by treatment of 15 μM albumin with 10 mM *p*-nitrophenyl acetate at pH 8.0, 22 °C for 48 h. The Mascot search engine matched 215 peptides to albumin in accession gi:3212456, yielding 76% coverage in the tryptic digest, and matched 331 peptides for 85% coverage in the GluC digest. Three peptides from a pepsin digest are included in supplemental Table S1. MS/MS spectra were manually evaluated before a labeled peptide was included in supplemental Table S1. MS/MS spectra positively identified acetylation of 59 lysines, 10 serines, 8 threonines, 4 tyrosines, and the N-terminal aspartate. Albumin has 59 lysines; every lysine was at least partially acetylated. The 10 acetylated serines were Ser-5, Ser-65, Ser-192, Ser-202, Ser-287, Ser-312, Ser-419, Ser-427, Ser-435, and Ser-454. The 8 acetylated threonines were Thr-68, Thr-76, Thr-79, Thr-83, Thr-467, Thr-474, Thr-527, and Thr-540. The four acetylated tyrosines were Tyr-84, Tyr-161, Tyr-401, and Tyr-411. The N-terminal aspartate Asp-1 was acetylated. Tyrosine 138, the ligand binding site in subdomain IB, which is modified by metabolites of polycyclic hydrocarbons (27) and by nitric oxide (28), was not modified by *p*-nitrophenyl acetate.

Both chemical and *in vivo* ε-*N*-acetylation of lysines has precedent (26, 29–32). Chemical *O*-acetylation of tyrosines also has precedent (33). Reports on acetylation of serines and threonines are more rare, though indirect evidence has been reported for bovine growth hormone-treated with acetic anhydride (34).

The peptides in supplemental Table S1 do not have an acetylated Lys at the C terminus, with the exception of Lys-162. The vast majority of acetylated lysines appear as missed cleavages within the peptide. This confirms reports in the literature (29) that trypsin generally does not recognize acetylated lysine as a cleavage site.

Carbamylation by ammonium cyanate, a degradation product of urea, would add a mass of +43, a value close to the mass of +42 from acetylation. To avoid carbamylation artifacts, we did not use urea in our protocol.

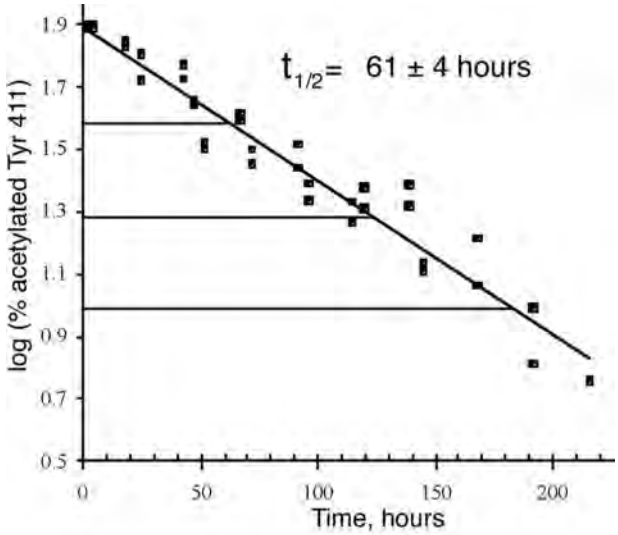

FIGURE 3. **Deacetylation rate of Tyr-411.** At time 0, Tyr-411 was acetylated 80%. After 61 h at pH 8.0, 22 °C, Tyr-411 was acetylated 40%. Deacetylation was monitored for 3.5 half-lives until only 5% of Tyr-411 was still acetylated. The three *horizontal lines* mark 3 half-lives. % acetylation was calculated from cluster areas in the MALDI-TOF mass spectrometer for peptides VRYTKKVPQVSTPTL and LVRYTKKVPQVSTPTL.

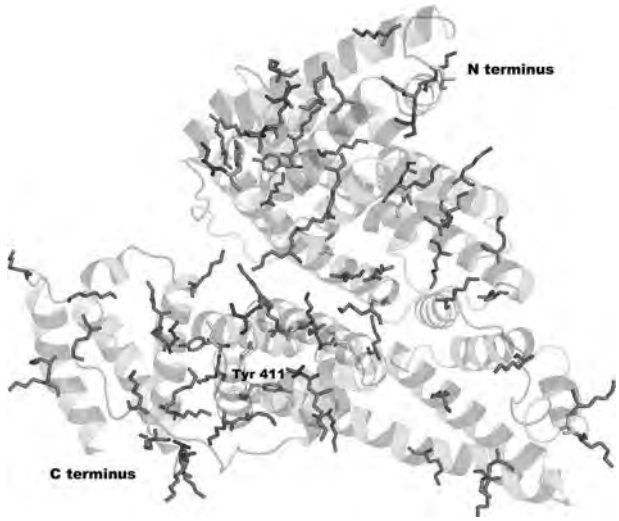

FIGURE 4. **Surface location of acetylated residues in human albumin.** The crystal structure of human albumin (Protein Data Bank code 1bm0) shows side-chains for acetylated lysines, tyrosines, serines, and threonines. Asp-1 and Lys-4 are missing from the structure (41).

Lysines Are Stably Acetylated—An estimate of the stability of acetylated lysines was obtained by measuring % acetylation of Lys-225 in peptide SQRFPK*AEF with time. After 6 h of reaction of 15 μM albumin with 0.5 mM *p*-nitrophenyl acetate, 9% of Lys-225 was acetylated. No change in % acetylation of this peptide was observed for up to 9 days. The lack of an increase in % acetylation with time is explained by the fact that 90% of the *p*-nitrophenyl acetate had been consumed after 6 h. The absence of a loss in % acetylation supports the conclusion that acetylated lysine is stable at pH 8.0. A peptide with 9% acetylated lysines was chosen for this example to make the point that a particular residue may be only partially acetylated.

A second example of stable acetylation was obtained by MALDI-TOF analysis of peptide D*AHK*S*EVAHRFK*DLGEENF. After 48 h reaction of 15 μM albumin with 10 mM *p*-nitrophenyl acetate, followed by dialysis to remove excess reagent, 100% of the N terminus (Asp-1), 100% of Lys-12, 78% of Lys-4, and 5% of Ser-5 were acetylated. After 18 days, the % acetylations were unchanged. We conclude that acetylated lysines, as well as the acetylated N terminus and acetylated serine, are stable at pH 8.0 and 22 °C.

Deacetylation of Tyr-411—Treatment of 15 μM albumin with 15 μM *p*-nitrophenyl acetate in 10 mM Tris-Cl, pH 8.0 resulted in exclusive acetylation of Tyr-411. No other residues were significantly acetylated. Tyr-411 was maximally acetylated after 1 h. Fig. 3 shows the maximum acetylation level of 80% and loss of the acetyl group with time. The half-life for deacetylation of Tyr-411 at pH 8.0, 22 °C was 61 ± 4 h. This very slow deacetylation rate ($k = 0.0002\ min^{-1}$) confirms a previous report that deacetylation is the rate-limiting step for reaction of human albumin with *p*-nitrophenyl acetate (14). On the other hand, turnover with *o*-nitrotrifluoroacetanilide is explained by the instability of the trifluoroacetyl adduct due to the electronic effect of the fluorine atoms (14).

Location of Ester-reactive Residues—The location of the reactive residues in the crystal structure of human albumin is shown in Fig. 4. All acetylated lysines, serines, and threonines are exposed to the surface. Tyr-411 is in a pocket 4.5 Å from Arg-410. Acetylated Tyr-84, -161, and -401 may also have been activated by interaction with nearby arginines.

Time to Complete Hydrolysis of 0.5 mM p-Nitrophenyl Acetate—Six hours after addition of *p*-nitrophenyl acetate to 15 μM albumin in 10 mM Tris-Cl, pH 8.0, 22 °C, 90% of the 0.5 mM *p*-nitrophenyl acetate had been consumed, and the rate of *p*-nitrophenol production by albumin had slowed so it was indistinguishable from the rate by buffer alone. By 24 h, the end point absorbance of 8.42, at 400 nm, was reached, and no further change in absorbance was obtained. The theoretical end point calculated from the extinction coefficient of 16,900 M^{-1} cm^{-1} for *p*-nitrophenolate ion at pH 8.0 was 8.45 (1), a value in close agreement with the observed end point.

Percent Acetylated Lysines—About 50% of the hydrolysis of 0.5 mM *p*-nitrophenyl acetate was due to reaction with 15 μM albumin, and about 50% to reaction with pH 8.0 buffer. Assuming that the 250 μM *p*-nitrophenyl acetate that reacted with 15 μM albumin resulted in stable acetylation of albumin, one can calculate about 16–17 molar equivalents of acetate bound per mol of albumin. If 16 of 59 lysines are acetylated, then on average 27% of each lysine was acetylated. This value is close to the 20–25% acetylation calculated for Lys-414 by MALDI-TOF analysis.

A third method was used to calculate % acetylated lysines. This method is based on the principle that lysines labeled with fluorodinitrobenzene are relatively stable to acid hydrolysis, whereas acetylated lysines are deacetylated to free lysines. Standard acid hydrolysis followed by amino acid composition analysis allows an estimate of the number of acetylated lysines (30). It was found that 27% of the lysines were acetylated in albumin treated with 0.5 mM *p*-nitrophenyl acetate, while 67% of the lysines were acetylated in albumin treated with 10 mM *p*-nitrophenyl acetate.

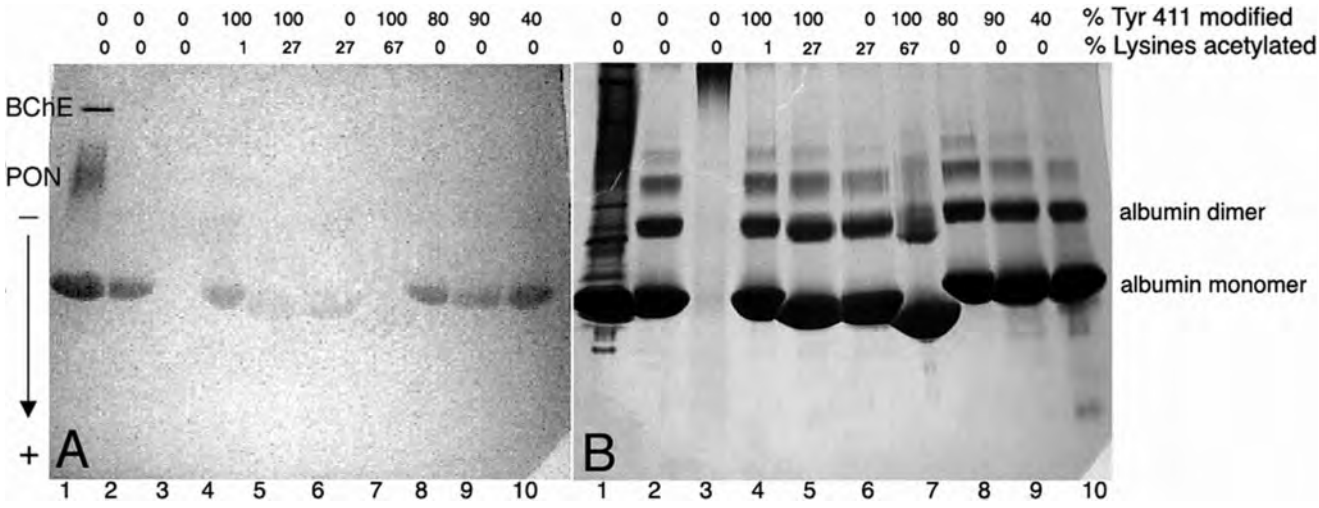

FIGURE 5. **Nondenaturing gel stained for esterase activity (*A*) and counterstained with Coomassie Blue (*B*).** *Lane 1*, 5 μl of human serum where the esterase bands are butyrylcholinesterase (*BChE*), paraoxonase (*PON*), and albumin. *Lane 2*, 200 μg of 99% pure fatty acid-free human albumin. *Lane 3*, 200 μg of denatured, carbamidomethylated albumin. *Lane 4*, 200 μg of albumin treated 5 min with 0.5 mM *p*-nitrophenyl acetate to acetylate 100% of Tyr-411. *Lane 5*, 200 μg of albumin treated with 0.5 mM *p*-nitrophenyl acetate for 6 h. *Lane 6*, 200 μg of albumin treated with 0.5 mM *p*-nitrophenyl acetate for 2 weeks. *Lane 7*, 200 μg of albumin treated with 10 mM *p*-nitrophenyl acetate for 48 h. *Lane 8*, 200 μg of albumin treated with DFP to label 80% of Tyr-411. *Lane 9*, 200 μg of albumin treated with chlorpyrifos oxon to label 90% of Tyr-411. *Lane 10*, 200 μg of albumin treated with chlorpyrifos oxon to label 40% of Tyr-411. The percent labeling of Tyr-411 was estimated from cluster areas of peaks in the MADLI-TOF mass spectrometer. The percent labeling of lysines was estimated from amino acid composition analysis. The *arrow* indicates the direction of migration of proteins on the gel.

Esterase Activity of Albumin—The esterase activity of human albumin can be visualized on a nondenaturing gel stained for esterase activity with β-naphthyl acetate and Fast Blue RR. The β-naphthol reacts with the diazonium salt of Fast Blue RR to produce a pink, insoluble azodye, which precipitates at the site where naphthol is released.

The gel in Fig. 5*A* shows naphthol production by the albumin present in 5 μl of human plasma (*lane 1*), as well as by 200 μg of pure human albumin (*lane 2*). Additional esterase bands in lane 1 are from butyrylcholinesterase and paraoxonase. The question of interest was how much of the apparent esterase activity of albumin was due to Tyr-411 and how much to irreversible acetylation of lysines? To answer this question, albumin preparations with various percent acetylation of Tyr-411 and lysines were loaded on the gel. The esterase activity in lane 4 where 100% of Tyr-411 was acetylated, was similar to that in lane 2 where none of the Tyr-411 was acetylated. The esterase activity in lane 5 where 100% of the Tyr-411 was acetylated, and 27% of the lysines were acetylated, was substantially decreased. The albumin in lane 6 was acetylated only on lysines, because Tyr-411 had completely deacetylated during 2 weeks incubation; the esterase activity in lane 6 was similar to that in lane 5, showing that lysines contributed more to albumin esterase activity than Tyr-411. The albumin in lane 7 was maximally acetylated by 10 mM *p*-nitrophenyl acetate, corresponding to the 82 residues acetylated in supplemental Table S1. The esterase activity in lane 7 was nearly abolished. Blocking Tyr-411 by covalent modification with DFP (lane 8) or chlorpyrifos oxon (lanes 9 and 10) had little effect on the apparent esterase activity.

The gel was counterstained with Coomassie Blue in Fig. 5*B* to show that protein loading per lane was equivalent. Slower migrating bands are consistent with higher molecular weight forms of albumin. Pure albumin is predominantly monomeric, but also forms dimers, and higher multimers. Fig. 5*B* clearly shows that the highly acetylated albumin (67%) in lane 7 migrated substantially further than native albumin in lane 2. Albumin that was 27% acetylated migrated slightly further than native. This behavior is consistent with elimination of positive charge from the protein by acetylation of the ε-*N*-amino groups of the lysines, thus giving the acetylated protein a greater net negative charge so that it would be attracted more readily to the positively charged electrode at the bottom of the gel.

The esterase activity of albumin is characterized by an initial burst of product formation that is equal to one equivalent of albumin, followed by slower formation of multiple equivalents of product (1). The fast phase has been attributed to initial acetylation of Tyr-411 (21), which is consistent with the rapid labeling of Tyr-411 described in Fig. 1. From the activity shown in Fig. 5, it can be concluded that the apparent slow phase esterase activity of albumin is due to release of *p*-nitrophenol upon acetylation of 59 lysines, and to some extent the surface accessible serines, threonines, and other tyrosines. This myriad of acetylations creates the appearance of enzymatic turnover, but it is not a true turnover process. Lysine, serine, and threonine are acetylated but do not release the acetate. Acetylated Tyr-411 does release the bound acetate (that is, it turns over), but the release rate is too slow to account for a significant part of the apparent esterase activity of albumin in a 30-min assay. Albumin must therefore be regarded as a pseudo-esterase, not an enzymatic esterase.

DISCUSSION

Albumin Esterase Activity—In our previous reports we had identified Tyr-411 as the residue in albumin that is labeled by soman, sarin, DFP, chlorpyrifos oxon, FP-biotin, and dichlorvos (16, 18). Others have also identified tyrosine as the site of covalent binding of soman, sarin, cyclosarin, and tabun to albumin (20). Organophosphorus agents inhibit the esterase activity of butyrylcholinesterase and other serine esterases by covalent binding to the active site serine (35). By analogy, we had

expected that OP binding to albumin would inhibit the esterase activity of albumin because it is commonly assumed that Tyr-411 is the active site residue on albumin that is responsible for esterase activity. The burst activity of albumin with *p*-nitrophenyl acetate is indeed inhibited by labeling Tyr-411 with an organophosphorus agent (21). However our results show that the slow steady state esterase activity of albumin is not inhibited by binding OP to Tyr-411, but instead is inhibited by acetylation of lysines. These results lead to a revised model of the esterase activity of albumin.

In this revised model of the esterase activity of albumin up to 82 residues participate. The most reactive residue is Tyr-411. Deacetylation of Tyr-411 occurs with a half-life of 61 ± 4 h, which means the *p*-nitrophenolate product formed in a 30-min reaction cannot come from turnover on Tyr-411. The majority of the "esterase" activity of albumin is due to a half-reaction with lysines, serines, threonines, and tyrosines. These acetylated residues do not turnover but form stable adducts. At high *p*-nitrophenyl acetate concentration a total of 59 lysines, 10 serines, 8 threonines, 4 tyrosines, and the N-terminal aspartate are acetylated.

The lysines of albumin are acetylated by *p*-nitrophenyl acetate, but are not labeled by organophosphorus esters. We have found no evidence for labeling of lysines by organophosphorus agents.

Confirmation of Means and Bender—When Means and Bender (1) found stable incorporation of [^{14}C]acetate into albumin they concluded that accelerated formation of *p*-nitrophenolate ion in the presence of serum albumin was not due to increased hydrolysis, but to rapid acetylation of serum albumin by *p*-nitrophenyl acetate. Means and Bender did not identify the residues involved and therefore their conclusion has been overlooked during the past 30 years while investigators focused on Tyr-411 as the esteratic site (36). With the availability of mass spectrometry we have identified the specific residues in albumin that become acetylated by *p*-nitrophenyl acetate and therefore provide proof for the concept introduced by Means and Bender to explain albumin esterase activity. Our results support their conclusion that the overall reaction rate, as reflected by appearance of *p*-nitrophenolate ion, corresponds to the sum of a large number of simultaneous reactions at different sites on the protein plus spontaneous hydrolysis.

Additional support for the conclusion that lysines and tyrosine participate in the esterase activity of albumin comes from studies of chemically modified albumin (7). Modification of tyrosine and lysine suppressed hydrolysis of carprofen glucuronide, leading to the conclusion that Tyr and Lys have roles in hydrolysis.

Significance—It is expected that other esters will also acetylate albumin. Acetyl salicylic acid (aspirin) has been shown to acetylate up to 3 residues on human albumin, though only one acetylation site has been identified to date, namely Lys-199 (37–39). Though acetylation rates must be very slow, the high concentration of albumin in plasma (0.6 mM) makes these reactions pharmacologically relevant.

Albumin is unusual in the family of plasma proteins because it has no carbohydrates. This makes the amino acids of albumin more accessible to acetylation than those of a protein like butyrylcholinesterase whose surface is sugar-coated. Nevertheless, it is possible that other proteins including butyrylcholinesterase are acetylated by carboxylic acid esters on multiple sites. Several lysines on ubiquitin are acetylated by aspirin (26). In some cases chemical acetylation has an important physiological consequence. For example, the anti-inflammatory action of aspirin is explained by acetylation of the N-terminal serine of prostaglandin synthetase (40).

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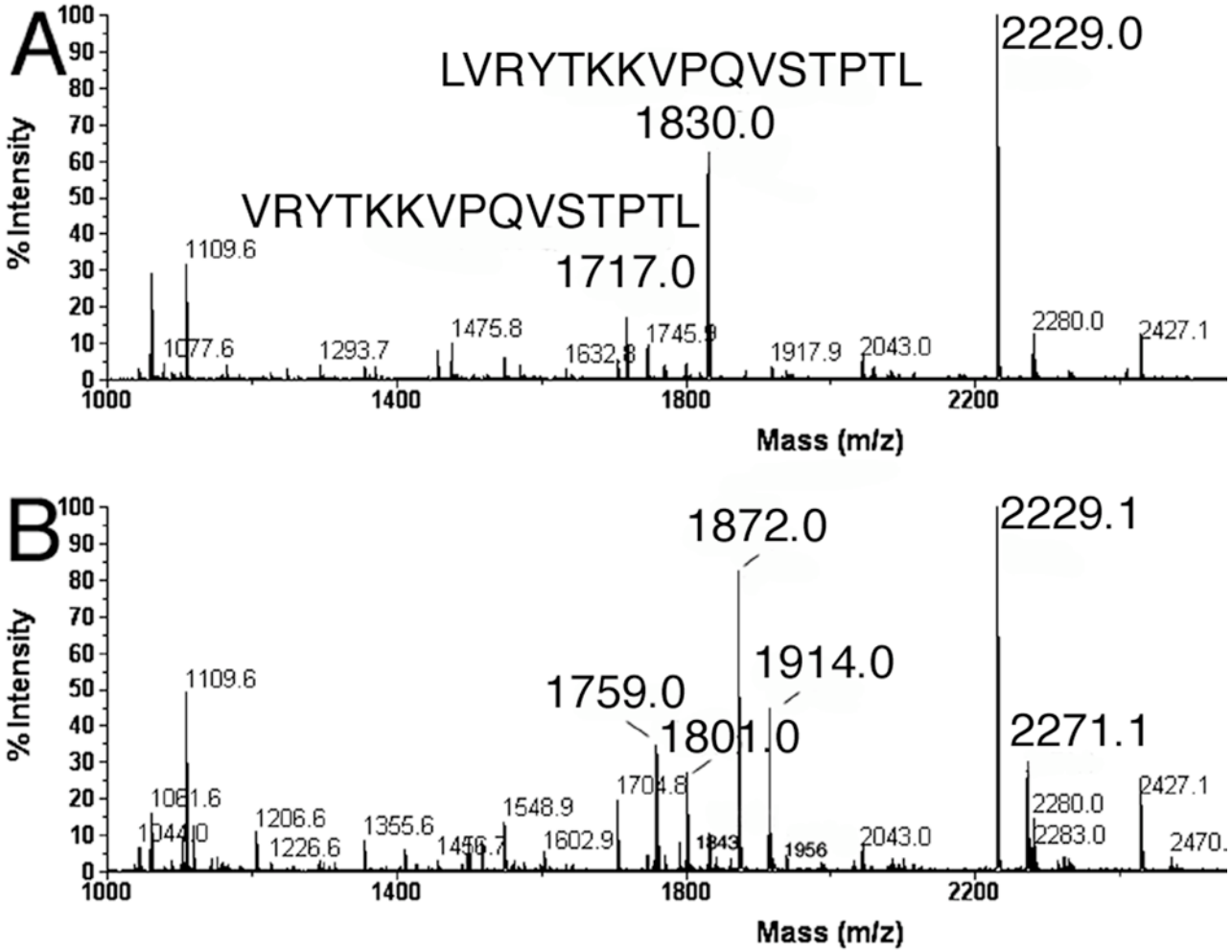

Figure S1. MALDI-TOF mass spectra of pepsin digested human albumin (15 μM) before (A) and after (B) treatment with 0.5 mM p-nitrophenyl acetate for 6 h. The 1717 amu peak for VRYTKKVPQVSTPTL in panel A has acquired +42, +84, and +126 amu masses at 1759, 1801, and 1843 amu in panel B. The 1830 amu peak for LVRYTKKVPQVSTPTL in panel A has acquired +42, +84, and +126 amu masses in panel B at 1872, 1914, and 1956 amu.

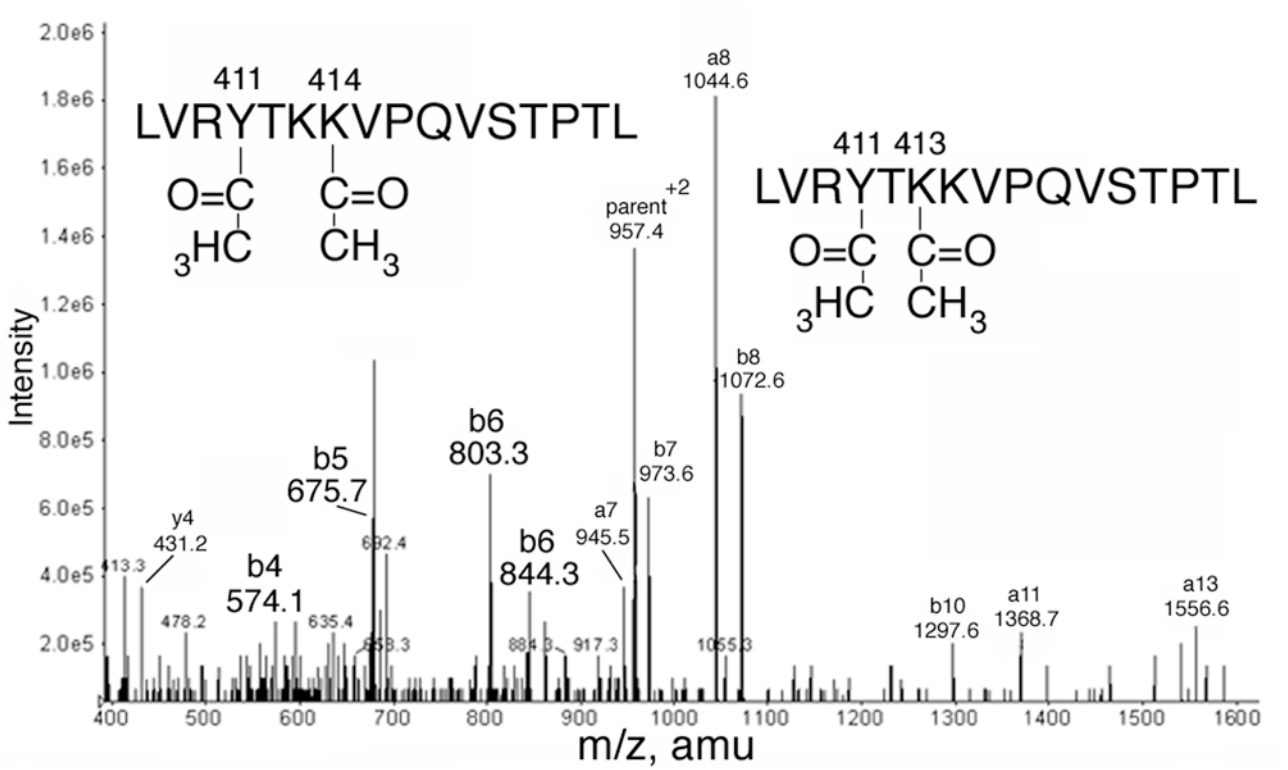

Figure S2. MS/MS spectrum to identify acetylated sites in LVRYTKKVPQVSTPTL after 6 h reaction of 1 mg/ml albumin with 0.5 mM p-nitrophenyl acetate. The doubly charged parent ion at 957.4 m/z, representing the singly charged 1914 amu ion, carries two acetyl groups. The acetyl groups are distributed between Y411/K414 and Y411/K413. The dominant ion is acetylated on Y411 and K414, while a less abundant ion is acetylated on Y411 and K413.

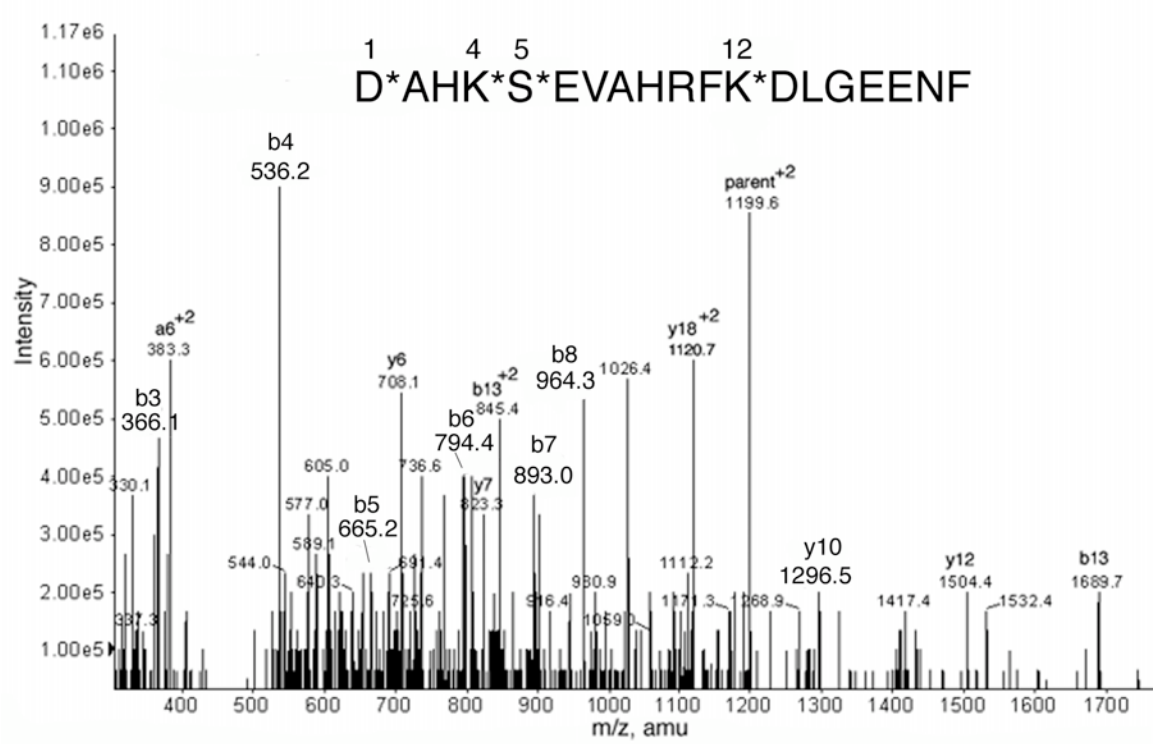

Figure S3. MS/MS spectrum to identify 4 acetylation sites on DAHKSEVAHRFKDLGEENF. Human albumin (15 μM) was incubated with 10 mM p-nitrophenyl acetate for 48 h at pH 8.0, before digestion with pepsin. The 4 acetylated residues in ion 2397 are D1, K4, S5, and K12.

Table S1. Mass spectrometry identification of residues in human albumin (accession number gi: 3212456) acetylated by 10 mM p-nitrophenyl acetate.

Start - End	Mr	Sequence	Acetylated	Protease	missed cleavages
1 - 19	2396.06	DAHKSEVAHRFKDLGEENF	D1 K4 S5 K12	pepsin	4
1 - 20	2482.19	DAHKSEVAHRFKDLGEENFK	D1 K4 K12	trypsin	3
7 - 17	1341.67	VAHRFKDLGEE	K12	Glu-C	1
11 - 20	1267.61	FKDLGEENFK	K12	trypsin	1
18 - 45	3417.74	NFKALVLIAFAQYLQQCPFEDHVKLVNE	K20 K41	Glu-C	1
18 - 45	3375.73	NFKALVLIAFAQYLQQCPFEDHVKLVNE	K41	Glu-C	1
42 - 64	2670.18	LVNEVTEFAKTCVADESAENCDK	K51	trypsin	1
49 - 57	1081.47	FAKTCVADE	K51	Glu-C	0
49 - 60	1368.59	FAKTCVADESAE	K51	Glu-C	1
61 - 82	2661.29	NCDKSLHTLFGDKLCTVATLRE	K64 K73	Glu-C	0
61 - 82	2619.28	NCDKSLHTLFGDKLCTVATLRE	K73	Glu-C	0
61 - 82	2661.29	NCDKSLHTLFGDKLCTVATLRE	S65 K73	Glu-C	0
65 - 81	2015.05	SLHTLFGDKLCTVATLR	S65 K73	trypsin	1
65 - 81	2015.05	SLHTLFGDKLCTVATLR	T68 K73	trypsin	1
65 - 81	1973.04	SLHTLFGDKLCTVATLR	T76	trypsin	1
65 - 81	1973.04	SLHTLFGDKLCTVATLR	K73	trypsin	1
74 - 98	3171.36	LCTVATLRETYGEM$_{OX}$ADCCAKQEPER	T76 T79 T83 K93	trypsin	2
74 - 98	3197.38	LCTVATLRETYGEMADCCAKQEPER	T76 T79 T83 Y84 K93	trypsin	2
82 - 98	2114.83	ETYGEMADCCAKQEPER	K93	trypsin	1
87 -100	1778.70	MADCCAKQEPERNE	K93	Glu-C	2
101 - 119	2389.20	CFLQHKDDNPNLPRLVRPE	K106	Glu-C	0
133 - 141	1245.66	TFLKKYLYE	K137	Glu-C	0

133 - 141	1287.68	TFLKKYLYE	K136 K137	Glu-C	0
137 - 144	1096.59	KYLYEIAR	K137	trypsin	1
146 - 162	2315.18	HPYFYAPELLFFAKRYK	K159 Y161 K162	trypsin	2
154 - 167	1745.95	LLFFAKRYKAAFTE	K159	Glu-C	0
154 - 167	1745.95	LLFFAKRYKAAFTE	Y161	Glu-C	0
154 - 167	1787.96	LLFFAKRYKAAFTE	Y161 K162	Glu-C	0
154 - 167	1787.96	LLFFAKRYKAAFTE	K159 K162	Glu-C	0
161 - 174	1703.73	YKAAFTECCQAADK	K162	trypsin	1
161 - 181	2499.16	YKAAFTECCQAADKAACLLPK	K162 K174	trypsin	2
161 - 186	3167.51	YKAAFTECCQAADKAACLLPKLDELR	K162 K174 K181	trypsin	3
163 - 181	2165.99	AAFTECCQAADKAACLLPK	K174	trypsin	1
168 - 184	2003.91	CCQAADKAACLLPKLDE	K174	Glu-C	0
168 - 184	2045.92	CCQAADKAACLLPKLDE	K174 K181	Glu-C	0
168 - 188	2517.17	CCQAADKAACLLPKLDELRDE	K174	Glu-C	1
168 - 188	2559.18	CCQAADKAACLLPKLDELRDE	K174 K181	Glu-C	1
175 - 186	1439.78	AACLLPKLDELR	K181	trypsin	1
175 - 195	2313.20	AACLLPKLDELRDEGKASSAK	K190	trypsin	3
182 - 197	1885.95	LDELRDEGKASSAKQR	K190 K195	trypsin	3
182 - 197	1927.96	LDELRDEGKASSAKQR	K190 S192 K195	trypsin	3
189 - 208	2319.20	GKASSAKQRLKCASLQKFGE	K190 K195 K199	Glu-C	0
189 - 208	2361.21	GKASSAKQRLKCASLQKFGE	K190 K195 K199 K205	Glu-C	0
198 - 209	1519.78	LKCASLQKFGER	K199 K205	trypsin	2
198 - 209	1561.79	LKCASLQKFGER	K199 S202 K205	trypsin	2
210 - 218	1060.58	AFKAWAVAR	K212	trypsin	1
223 - 233	1293.66	FPKAEFAEVSK	K225	trypsin	1
223 - 240	2106.12	FPKAEFAEVSKLVTDLTK	K225 K233	trypsin	2
234 - 257	2898.29	LVTDLTKVHTECCHGDLLECADDR	K240	trypsin	1
245 - 266	2725.12	CCHGDLLECADDRADLAKYICE	K262	Glu-C	1
253 - 266	1740.74	CADDRADLAKYICE	K262	Glu-C	0
258 - 274	1982.93	ADLAKYICENQDSISSK	K262	trypsin	1
258 - 276	2266.12	ADLAKYICENQDSISSKLK	K262 K274	trypsin	2
263 - 276	1725.82	YICENQDSISSKLK	K274	trypsin	1
267 - 277	1289.65	NQDSISSKLKE	K274	Glu-C	0
267 - 277	1331.66	NQDSISSKLKE	K274 K276	Glu-C	0
275 - 286	1629.81	LKECCEKPLLEK	K276 K281	trypsin	2
286 - 292	927.41	KSHCIAE	K286 S287	Glu-C	0
312 - 321	1296.57	SKDVCKNYAE	S312 K317	Glu-C	0
312 - 321	1296.57	SKDVCKNYAE	K313 K317	Glu-C	0
318 - 336	2341.11	NYAEAKDVFLGMFLYEYAR	K323	trypsin	1
322 - 333	1473.72	AKDVFLGMFLYE	K323	Glu-C	0
349 - 359	1337.71	LAKTYETTLEK	K351	trypsin	1
349 - 372	2913.30	LAKTYETTLEKCCAAADPHECYAK	K351 K359	trypsin	2
359 - 376	2211.90	KCCAAADPHECYAKVFDE	K359	Glu-C	1
359 - 376	2253.91	KCCAAADPHECYAKVFDE	K359 K372	Glu-C	1
369 - 376	1072.45	CYAKVFDE	K372	Glu-C	0
377 - 393	2127.07	FKPLVEEPQNLIKQNCE	K378	Glu-C	2
377 - 393	2169.08	FKPLVEEPQNLIKQNCE	K378 K389	Glu-C	2
390 - 410	2640.30	QNCELFEQLGEYKFQNALLVR	K402	trypsin	1
390 - 410	2640.30	QNCELFEQLGEYKFQNALLVR	Y401	trypsin	1
401 - 425	3047.67	YKFQNALLVRYTKKVPQVSTPTLVE	Y401 K402 K413	Glu-C	0
408 - 423	1997.08	LVRYTKKVPQVSTPTL	Y411 K413 K414 S419	pepsin	2
409 - 423	1883.99	VRYTKKVPQVSTPTL	Y411 K413 K414 S419	pepsin	1
411 - 428	2115.16	YTKKVPQVSTPTLVEVSR	K413 K414	trypsin	2
411 - 428	2157.17	YTKKVPQVSTPTLVEVSR	K413 K414 S419	trypsin	2
414 - 428	1680.94	KVPQVSTPTLVEVSR	K414	trypsin	1
414 - 428	1722.95	KVPQVSTPTLVEVSR	K414 S419	trypsin	1
426 - 442	2039.00	VSRNLGKVGSKCCKHPE	K432 K436	Glu-C	0

426 - 442	2081.01	VSRNLGKVGSKCCKHPE	K432 K436 K439	Glu-C	0
426 - 442	2081.01	VSRNLGKVGSKCCKHPE	S427 K436 K439	Glu-C	0
426 - 442	2123.03	VSRNLGKVGSKCCKHPE	K432 S435 K436 K439	Glu-C	0
429 - 439	1333.65	NLGKVGSKCCK	K432 K436	trypsin	2
429 - 444	1937.95	NLGKVGSKCCKHPEAK	K432 K436 K439	trypsin	3
440 - 466	3293.60	HPEAKRM_{OX}PCAEDYLSVVLNQLCVLHEK	K444	trypsin	2
446 - 466	2575.21	M_{OX}PCAEDYLSVVLNQLCVLHEK	S454	trypsin	0
446 - 472	3214.55	MPCAEDYLSVVLNQLCVLHEKTPVSDR	K466	trypsin	1
446 - 472	3230.54	M_{OX}PCAEDYLSVVLNQLCVLHEKTPVSDR	T467	trypsin	1
446 - 472	3256.56	MPCAEDYLSVVLNQLCVLHEKTPVSDR	S454 K466	trypsin	1
466 - 479	1721.81	KTPVSDRVTKCCTE	T467	Glu-C	0
466 - 479	1763.82	KTPVSDRVTKCCTE	K466 K475	Glu-C	0
473 - 484	1507.71	VTKCCTESLVNR	K475	trypsin	1
473 - 484	1549.72	VTKCCTESLVNR	T474 K475	trypsin	1
496 - 505	1238.58	TYVPKEFNAE	K500	Glu-C	1
501 - 521	2586.17	EFNAETFTFHADICTLSEKER	K519	trypsin	1
506 - 520	1839.83	TFTFHADICTLSEKE	K519	Glu-C	1
521 - 531	1396.80	RQIKKQTALVE	K524 K525	Glu-C	0
522 - 534	1580.95	QIKKQTALVELVK	K524 K525	trypsin	2
526 - 534	1041.61	QTALVELVK	T527	trypsin	0
532 - 542	1403.81	LVKHKPKATKE	K534 K538 K541	Glu-C	0
535 - 557	2798.43	HKPKATKEQLKAVMDDFAAFVEK	K536 K538 T540 K545	trypsin	4
539 - 557	2224.11	ATKEQLKAVMDDFAAFVEK	K541 K545	trypsin	2
539 - 560	2772.28	ATKEQLKAVMDDFAAFVEKCCK	T540 K541 K545 K557	trypsin	3
543 - 556	1624.78	QLKAVMDDFAAFVE	K545	Glu-C	0
546 - 560	1847.79	AVMDDFAAFVEKCCK	K557	trypsin	1
558 - 574	2158.90	CCKADDKETCFAEEGKK	K560 K564	trypsin	3
558 - 574	2200.91	CCKADDKETCFAEEGKK	K560 K564 K573	trypsin	3
561 - 573	1540.64	ADDKETCFAEEGK	K564	trypsin	1
561 - 574	1710.74	ADDKETCFAEEGKK	K564 K573	trypsin	2
561 - 585	2705.32	ADDKETCFAEEGKKLVAASQAALGL	K573 K574	trypsin	3
565 - 585	2276.14	ETCFAEEGKKLVAASQAALGL	K573 K574	trypsin	2
572 - 585	1367.81	GKKLVAASQAALGL	K573	Glu-C	0
572 - 585	1409.82	GKKLVAASQAALGL	K573 K574	Glu-C	0
574 - 585	1182.70	KLVAASQAALGL	K574	trypsin	1

The added mass from acetate is 42, and from carbamidomethylation is 57. All 59 lysines are acetylated. 10 serines are acetylated: S5, S65, S192, S202, S287, S312, S419, S427, S435, S454. 8 threonines are acetylated: T68, T76, T79, T83, T467, T474, T527, T540. 4 tyrosines are acetylated: Y84, Y161, Y401, Y411. Each residue listed in the "acetylated" column was unequivocally identified based on the presence of diagnostic b and/or y ions.

Five Tyrosines and Two Serines in Human Albumin Are Labeled by the Organophosphorus Agent FP-Biotin

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Tyrosine 411 of human albumin is an established site for covalent attachment of 10-fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide (FP-biotin), diisopropylfluorophosphate, chlorpyrifos oxon, soman, sarin, and dichlorvos. This work investigated the hypothesis that other residues in albumin could be modified by organophosphorus agents (OP). Human plasma was aggressively treated with FP-biotin; plasma proteins were separated into high and low abundant portions using a proteome partitioning antibody kit, and the proteins were digested with trypsin. The FP-biotinylated tryptic peptides were isolated by binding to monomeric avidin beads. The major sites of covalent attachment identified by mass spectrometry were Y138, Y148, Y401, Y411, Y452, S232, and S287 of human albumin. Prolonged treatment of pure human albumin with chlorpyrifos oxon labeled Y138, Y150, Y161, Y401, Y411, and Y452. To identify the most reactive residue, albumin was treated for 2 h with DFP, FP-biotin, chlorpyrifos oxon, or soman, digested with trypsin or pepsin, and analyzed by mass spectrometry. The most reactive residue was always Tyr 411. Diethoxyphosphate-labeled Tyr 411 was stable for months at pH 7.4. These results will be useful in the development of specific antibodies to detect OP exposure and to engineer albumin for use as an OP scavenger.

Introduction

Organophosphorus agents are used in agriculture as pesticides and are stocked by the military as chemical warfare agents. These chemicals are toxic to insects, fish, birds, and mammals. Seizures, respiratory arrest, and death are explained by a cascade of reactions that begins with inhibition of acetylcholinesterase. Although acetylcholinesterase in red blood cells and butyrylcholinesterase in plasma are established biomarkers of organophosphorus ester (OP)[1] exposure, additional biomarkers are being sought. Albumin has the potential to serve as a new biomarker of OP exposure (*1*, *2*). Albumin has been reported to covalently bind diisopropylfluorophosphate (DFP), sarin, soman, cyclosarin, tabun, 10-fluoroethoxyphosphinyl-*N*-biotinamido pentyldecanamide (FP-biotin), chlorpyrifos oxon (CPO), and dichlorvos (*2*−*6*) and to hydrolyze CPO, paraoxon (*7*, *8*), and *O*-hexyl *O*-2,5-dichlorophenyl phosphoramidate (*9*). Mass spectrometry has identified tyrosine 411 of human albumin as the site for covalent attachment of OP nerve agents and OP pesticides (*6*). A second site for covalent attachment of soman was suggested by experiments that found more fluoride ion released than could be accounted for by one site in albumin (*3*). Pretreatment of albumin with decanoate, a lipid that binds to the Tyr 411 subdomain, inhibited incorporation of 91% of ^{3}H-DFP, leaving open the possibility that 9% of the ^{3}H-DFP bound to other sites (*10*). Crystallization trials of CPO-labeled human albumin yielded gelatinous soft amorphous crystals, further suggesting the likelihood that more than one site was labeled and that labeling was not uniform. The goal of this study was to determine if sites in addition to Tyr 411 could make a covalent bond with OP and to identify the labeled residues.

Our starting premise was that OP bound exclusively to Tyr 411 of human albumin. For certain studies, we wanted 100% labeling on Tyr 411. Therefore, we treated albumin with excess CPO. The sample was checked by mass spectrometry to confirm the site of OP labeling, and to our surprise, we found several CPO-labeled peptides.

Our studies with human plasma were initiated with the goal of identifying OP-labeled proteins in human plasma. We had expected to identify several FP-biotin-labeled proteins. However, we found only FP-biotin-labeled albumin. The albumin was covalently modified on five tyrosines and two serines. FP-biotin was used for studies with plasma because biotinylated peptides are readily purified by binding to immobilized avidin beads (*1*, *11*).

Experimental Procedures

Materials. FP-biotin (MW 592.32) was custom synthesized in the laboratory of Dr. Charles M. Thompson at the University of Montana (Missoula, MT) (*12*). FP-biotin was dissolved in methanol and stored at −80 °C. CPO (ChemService Inc. West Chester, PA;

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[1] Abbreviations: CPO, chlorpyrifos oxon; DFP, diisopropylfluorophosphate; FP-biotin, 10-fluoroethoxyphosphinyl-*N*-biotinamido pentyldecanamide; OP, organophosphorus ester; LC/MS/MS, liquid chromatography tandem mass spectrometry; MALDI-TOF-TOF, matrix-assisted laser desorption tandem time-of-flight mass spectrometry.

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MET-674B) was dissolved in ethanol and stored at −80 °C. DFP, a liquid with a concentration of 5.73 M, was from Sigma (D0879). Soman from CEB (Vert-le-Petit, France) was dissolved in isopropanol. A Proteome Partitioning Kit, ProteomeLab IgY-12 High Capacity in Spin Column format contained IgY antibodies directed against the 12 most abundant proteins in human plasma (Beckman Coulter #A24331 S0510903) including albumin, IgG, fibrinogen, transferrin, IgA, IgM, HDL (apo A-I and apo A-II), haptoglobin, α-1-antitrypsin, α-1-acid glycoprotein, and α-2-macroglobulin. Cibacron blue 3GA agarose (Sigma, C1535) bound 10−20 mg of human albumin per mL of gel. Porcine trypsin (Promega, Madison, WI; V5113 sequencing grade modified trypsin) at a concentration of 0.4 μg/μL in 50 mM acetic acid was stored at −80 °C. Pepsin (Sigma, St. Louis, MO; P6887 from porcine gastric mucosa) was dissolved in 10 mM HCl to make a 1 mg/mL solution and stored at −80 °C. Monomeric avidin agarose beads (#20228) were from Pierce Co. NeutrAvidin agarose beads (#29202) were from Thermo Scientific (Rockford, IL). Human plasma (EDTA anticoagulant) was from an adult male, who had fasted overnight before donating blood. Fatty acid free human albumin (Fluka 05418) was from Sigma/Aldrich.

Procedures for FP-Biotin-Labeled Plasma. Separation of Low and High Abundance Proteins in Human Plasma. Two hundred microliters of human plasma were fractionated into low and high abundance proteins by processing 20 μL of plasma at a time on the Beckman Coulter Proteome IgY Spin column depletion kit. The yield of high abundance proteins was 4800 μg in 400 μL. Of this, 123 μL was labeled with FP-biotin, 123 μL was used as a negative control, and the remainder was used for determination of protein concentration.

High Abundance Proteins Labeled with FP-Biotin, Digested with Trypsin, and Purified on Monomeric Avidin. The high abundance fraction of plasma had albumin as its major component. A 123 μL aliquot of the high abundance fraction was treated with 1.25 μL of 20 mM FP-biotin for 48 h at 37 °C at pH 8.0. The final FP-biotin concentration was 200 μM. Excess FP-biotin was removed by dialysis against 2 × 4 L of 10 mM ammonium bicarbonate.

Proteins, in 8 M urea, were reduced with 5 mM dithiothreitol and alkylated with 40 mM iodoacetamide. The samples were diluted to reduce the concentration of urea to 2 M. Proteins were digested with a 1:50 ratio of trypsin to protein at 37 °C overnight. The trypsin was inactivated by heating the sample in a boiling water bath for 10 min. It was necessary to inactivate trypsin because trypsin could have destroyed the avidin protein used in the next step. FP-biotinylated peptides were purified by binding to 0.5 mL of monomeric avidin beads. Nonspecifically bound peptides were washed off with high salt buffers. The column was washed with water to remove salts, and FP-biotinylated peptides were eluted with 10% acetic acid. The eluate was dried in a vacuum centrifuge in preparation for mass spectrometry. The negative control was human plasma treated with everything except FP-biotin.

Depletion of Albumin on Cibacron Blue, Labeling with FP-Biotin, Digestion with Trypsin, and Purification on NeutrAvidin. An albumin-depleted plasma sample was prepared by binding 0.6 mL of human plasma to 2 mL of Cibacron Blue and collecting the protein that eluted in 10 mL of 10 mM TrisCl, pH 8.0, containing 0.3 M NaCl. About 70% of the albumin was removed from the plasma sample by this procedure. The protein was desalted, concentrated to 0.5 mL, and labeled with 100 μM FP-biotin at 37 °C for 16 h in 10 mM ammonium bicarbonate. The labeled protein was denatured in 8 M urea, reduced with dithiothreitol, carbamidomethylated with iodoacetamide, and desalted on a spin column. The yield was 2000 μg in 500 μL. The entire sample was digested with 40 μg of trypsin (Promega) at 37 °C overnight. The FP-biotinylated tryptic peptides were bound to 0.1 mL of NeutrAvidin beads, washed with high salt buffers and water, and eluted with 45% acetonitrile and 0.1% formic acid.

Mass Spectrometry on QSTAR Elite and QTRAP 2000. Five micrograms of the high abundance FP-biotinylated peptides purified with monomeric avidin beads was analyzed on the QSTAR elite liquid chromatography tandem mass spectrometry (LC/MS/MS) system with ProteinPilot 2.0 software at the Applied Biosystems laboratories (Framingham, MA).

A second 5 μg aliquot from the same protein preparation, a negative control sample, and the NeutrAvidin purified peptides were analyzed by LC/MS/MS on the QTRAP 2000 mass spectrometer (Applied Biosystems) at the University of Nebraska Medical Center with Analyst 1.4.1 software. The digest was dried in a vacuum centrifuge and dissolved in 5% acetonitrile and 0.1% formic acid to make 0.5 μg/μL. A 10 μL aliquot was injected into the HPLC nanocolumn (#218MS3.07515 Vydac C18 polymeric rev-phase, 75 μm i.d. × 150 mm long; P.J. Cobert Assoc, St. Louis, MO). Peptides were separated with a 90 min linear gradient from 0 to 60% acetonitrile at a flow rate of 0.3 μL/min and electrosprayed through a nanospray fused silica emitter (360 μm o.d., 75 μm i.d., 15 μm taper, New Objective) directly into the QTRAP 2000, a hybrid quadrupole linear ion trap mass spectrometer. An ion spray voltage of 1900 V was maintained between the emitter and the mass spectrometer. Information-dependent acquisition was used to collect MS, enhanced MS, and MS/MS spectra for the three most intense peaks in each cycle, having a charge of +1 to +4, a mass between 400 and 1700 *m*/*z*, and an intensity >10000 counts per s. All spectra were collected in the enhanced mode, that is, using the trap function. Precursor ions were excluded for 30 s after one MS/MS spectrum had been collected. The collision cell was pressurized to 40 μTorr with pure nitrogen. Collision energies between 20 and 40 eV were determined automatically by the software, based on the mass and charge of the precursor ion. The mass spectrometer was calibrated on selected fragments from the MS/MS spectrum of Glu-Fibrinopeptide B. MS/MS spectra were submitted to Mascot for identification of labeled peptides and amino acids (*13*). MASCOT identified FP-biotinylated Y*LYEIAR (score 17), HPY*FYAPELLFFAK (score 14), and MPCAEDY*LSVVLNQLCVLHEK (score 15) but none of the other FP-biotinylated peptides. The others were identified by manually searching the MS/MS data files using the Extracted Ion Chromatogram feature of the Analyst software. The scores were low because the software did not recognize the characteristic fragments of FP-biotin at 227, 312, and 329. It also did not recognize the 591 ion of FP-biotin or the FP-biotin-tyrosine immonium ions at 708 and 691 or fragments containing dehydroalanine in place of serine. The ions that Mascot did not recognize were often very intense.

The MASCOT modification file is an open source software called UNIMOD. The FP-biotin modification on serine, threonine, and tyrosine was introduced according to the instructions found on the Web site http://www.unimod.org. Access to the modification is freely available to all MASCOT users in the Variable Modifications menu under the name FP-biotin. Fragments of FP-biotin are not part of the MASCOT modification file. Peptides yielding FP-biotin fragments at 227, 312, and 329 amu were identified using the Extracted Ion Chromatogram feature of ABI's Analyst software. Neutral loss of fragments of FP-biotin were identified by manual inspection of MS/MS spectra.

Mass Spectrometry by Matrix-Assisted Laser Desorption Tandem Time-of-Flight Mass Spectrometry (MALDI-TOF-TOF) 4800. A 0.5 μL aliquot of essentially salt-free samples was spotted on a MALDI target plate, air-dried, and overlaid with 0.5 μL of 10 mg/mL α-cyano-4-hydroxy cinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid. MS spectra were acquired using a MALDI-TOF-TOF 4800 (Applied Biosystems), with a laser power of 3000 V, in positive reflector mode. Each spectrum was the average of 500 laser shots. The mass spectrometer was calibrated against des-Arg-Bradykinin (904.468 Da), angiotensin 1 (1296.685 Da), Glu-Fibrinopeptide B (1570.677 Da), and neurotensin (1672.918 Da) (Cal Mix 1 from Applied Biosystems).

Procedures for Pure Albumin. Percent OP-Labeled Tyr 411 Monitored by MALDI-TOF. A 5 μL aliquot of 10 mg/mL albumin was diluted with 5 μL of 1% trifluoroacetic and digested with 2 μL of 1 mg/mL porcine pepsin for 1−2 h at 37 °C. The digest was diluted with 50% acetonitrile and 0.1% trifluoroacetic acid to give a final protein concentration of about 0.5 mg/mL. A 0.5 μL aliquot was spotted on the MALDI target plate, dried, and

overlaid with 0.5 μL of 10 mg/mL α-cyano-4-hydroxy cinnamic acid. MS spectra were acquired with the laser set at 3000 V and were saved to Data Explorer. When the saved spectrum was opened in Data Explorer, the cluster areas appeared in an output window. Percent OP-labeled Tyr 411 was calculated by dividing the cluster area of the labeled peptide by the sum of the cluster areas for the unlabeled and labeled peaks. The unlabeled peptides were ^{409}VRYT-KKVPQVSTPTL^{423} (1717.0 amu) and ^{408}LVRYTKKVPQ-VSTPTL^{423} (1830.1 amu). After covalent bond formation with CPO, these masses increased by 136 amu to become 1853.0 and 1966.1 amu. After covalent bond formation with FP-biotin, these masses increased by 572.3 to become 2289.3 and 2402.4 amu.

Prolonged Treatment of Albumin with CPO. At the time that we prepared CPO-labeled human albumin, we knew that Tyr 411 was labeled by CPO and had no reason to suspect that other residues might also be labeled. CPO dissolved in ethanol was added to an albumin solution in 10 mM ammonium bicarbonate, pH 8.3, and 0.01% sodium azide in six additions over a 1 month period.

The labeling efficiency was poor when the albumin concentration was 500 mg/mL, so the albumin was diluted to 35 mg/mL, and then to 5 mg/mL, and finally to 1 mg/mL. The final ratio was 7.7 μmol of albumin to 146 μmol of CPO. During the 1 month labeling time, the decision to add more CPO was based on the percent Tyr 411 labeled. No further additions of CPO were made after 85% of the Tyr 411 had been labeled. The labeled albumin was dialyzed against 10 mM potassium phosphate, pH 7.0, and 0.01% azide and processed for LC/MS/MS analysis in the QTRAP mass spectrometer.

Identification of the Most Reactive Residues. The conditions reported to label 1 mol of albumin with 1 mol of DFP were used (*10*). Human albumin (1.8 mg/mL) in 10 mM TrisCl, pH 8.0, was treated with a 20-fold molar excess of DFP for 2 h at room temperature. The reaction was stopped by the addition of solid urea to 8 M and boiling for 10 min in the presence of 10 mM dithiothreitol. Free sulfhydryl groups were alkylated with iodoacetamide. The carbamidomethylated albumin was dialyzed against 2 × 4 L of 10 mM ammonium bicarbonate and digested with trypsin. Tryptic peptides were subjected to LC/MS/MS on the QTRAP 2000.

The experiment was repeated with FP-biotin, soman, and CPO. A 15 μM solution of albumin in 10 mM TrisCl pH 8.0 was treated with 150 μM FP-biotin or 150 μM soman or 150 μM CPO for 2 h at 22 °C. Samples with intact disulfides were digested with pepsin and analyzed by MALDI-TOF. Carbamidomethylated tryptic peptides were analyzed by LC/MS/MS. Tryptic peptides labeled with FP-biotin were also purified on monomeric avidin beads eluted with 50% acetonitrile and 0.1% formic acid and analyzed by MALDI-TOF-TOF.

Stability of CPO-Labeled Tyr 411. The stability of CPO-labeled Tyr 411 in human albumin was tested at pH 1.5, 7.4, and 8.3 after incubation for up to 7 months at 22 and −80 °C. Albumin labeled on 97% of Tyr 411 with diethoxyphosphate was prepared by incubating 1 mg/mL human albumin (15.6 μM) in 10 mM TrisCl, pH 8.0, and 0.01% sodium azide with 118 μM CPO for 2.5 days at 22 °C. Excess CPO was removed by dialysis of the 8.5 mL solution against 2 × 4 L of 10 mM ammonium bicarbonate, pH 8.3, and 0.01% azide.

pH 1.5. The pH of 2.6 mL of the dialyzed CPO-albumin was adjusted to pH 1.5 by adding 2.6 mL of 1% trifluoroacetic acid. Half of the sample was stored at room temperature, and half was divided into 40 μL aliquots and stored at −80 °C.

pH 7.4. The pH was adjusted to pH 7.4 by dialyzing 3.3 mL of the CPO-albumin preparation against 4.5 L of 10 mM potassium phosphate, pH 7.4, and 0.01% azide. To avoid freeze−thaw artifacts, samples intended for storage at −80 °C were divided into 20 μL aliquots so that each tube was thawed only once.

pH 8.3. The pH of 2.6 mL of CPO-albumin was brought to pH 8.3 by dialysis against 10 mM ammonium bicarbonate and 0.01% sodium azide, pH 8.3. Samples to be stored at −80 °C were divided into 65 tubes each containing 20 μL.

After various times, a tube was removed from −80 °C storage, and the entire contents were digested with pepsin. Samples stored at room temperature were also digested with pepsin. The digests were analyzed by MALDI-TOF, and % labeled Tyr 411 was calculated from cluster areas as described above.

The sample stored at −80 °C in pH 7.4 buffer was analyzed by LC/MS/MS to determine whether sites in addition to Tyr 411 were labeled. The CPO-albumin was denatured, reduced, carbamidomethylated, and digested with trypsin in preparation for LC/MS/MS. The diethoxyphosphate group was found on Tyr 411 and Tyr 138.

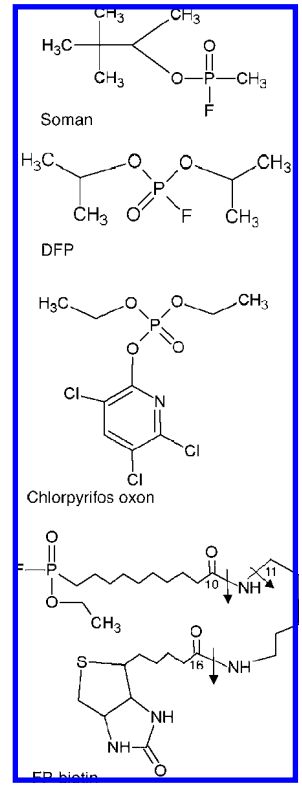

Figure 1. Structures of organophosphorus agents. Covalent binding to tyrosine or serine results in loss of the fluoride ion from soman, DFP, and FP-biotin and of the aromatic ring from CPO, so that the added mass is 162.2 for soman, 164.1 for DFP, 136.0 for CPO, and 572.3 for FP-biotin. The arrows in FP-biotin indicate fragmentation sites. A 227 amu ion is produced by cleavage between carbon 16 and the adjacent nitrogen. A 329 amu ion is produced by cleavage between carbon 10 and the adjacent nitrogen. The 312 amu ion is produced by loss of the amine from the 329 ion.

Results

FP-Biotin Labeled Albumin in Human Plasma. The structures of the organophosphorus agents are shown in Figure 1. Five tyrosines and two serines in human albumin were labeled with FP-biotin including Tyr 138, Tyr 148, Tyr 401, Tyr 411, Tyr 452, Ser 232, and Ser 287 (Table 1).

Supporting MS/MS spectra for these assignments are in Figures 2−6. A peptide labeled with FP-biotin had ions at 227, 312, and 329 atomic mass units (amu) resulting from fragmentation of FP-biotin (*12*). Two ions characteristic of covalent binding of FP-biotin to the hydroxyl group of tyrosine are the immonium ion of tyrosine-FP-biotin at 708 amu and its partner ion at 691 amu, produced by loss of NH_3. The 708 and 691 amu masses are prominent in Figure 2A,B but barely visible in Figure 3A,B. An additional complexity in Figure 2A is the presence of ions that had lost a 329 or 226 amu fragment from FP-biotin.

The masses in Figure 2A are consistent with the sequence YTK where the added mass of 572 amu from FP-biotin is on Tyr. The complete y-ion series is present (y1, 147.0 amu, Lys;

Table 1. FP-Biotinylated Human Albumin Tryptic Peptides Identified by LC/MS/MS[a]

start−end	M_r	sequence	FP-biotinylated
138−144	1499.8	Y*LYEIAR	Y138
146−159	2315.2	HPY*FYAPELLFFAK	Y148
226−233	1452.7	AEFAEVS*K	S232
287−313	3546.6	S*HCIAEVENDEMPADLPSLAAD-FVESK	S287
390−402	2230.0	QNCELFEQLGEY*K	Y401
411−413	983.5	Y*TK	Y411
446−466	3090.5	MPCAEDY*LSVVLNQLCVLHEK	Y452

[a] Residue numbers in accession # gi: 3212456 are for the mature albumin protein and do not include the 24 amino acid signal peptide. The added mass from FP-biotin is 572.3 amu. Cysteines were carbamidomethylated, thus adding a mass of 57 amu.

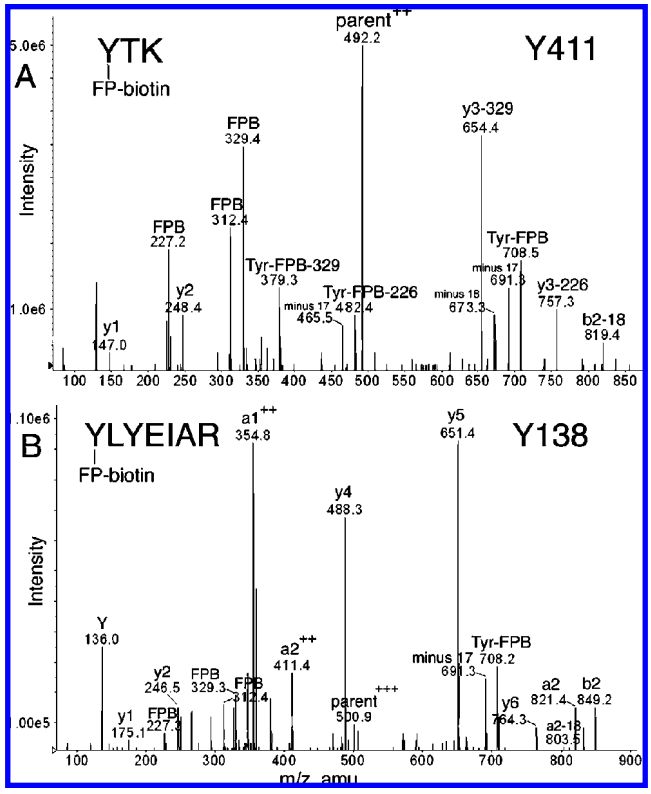

Figure 2. MS/MS spectra of albumin peptides labeled with FP-biotin on Tyrosine. (A) Tyr 411 in peptide YTK and (B) Tyr 138 in peptide YLYEIAR are covalently modified by FP-biotin. The characteristic fragments of FP-biotin at 227.2, 312.4, and 329.3 amu are present. Ions characteristic of FP-biotin modification on tyrosine are the immonium ion at 708 amu and the immonium ion minus 17 at 691 amu. Support for modification of the first tyrosine rather than the second in YLYEIAR is the mass of ions a2, b2, and $a2^{+2}$. The doubly charged parent ion in panel A had a mass of 492.2 amu. The triply charged parent ion in panel B had a mass of 500.9 *m*/*z*.

y2, 248.4 amu, LysThr; and the doubly charged, FP-biotinylated parent ion). Peaks at 227.2, 312.4, 329.4, 691.3, and 708.5 amu are indicative of the presence of FP-biotinylated tyrosine. The remaining major peaks are consistent with various FP-biotinylated tyrosine fragments missing pieces of the FP-biotin moiety.

Peptide YLYEIAR has two tyrosines. A y-ion series (y1−y6) indicates that the FP-biotin label is on the N-terminal Tyr. Additional evidence for labeling on Tyr 138 rather than on Tyr 140 was the presence of the a2 ion at 821.4 amu, the b2 ion at 849.2 amu, the $a1^{+2}$ ion at 354.8, and the $a2^{+2}$ ion at 411.4 *m*/*z* (Figure 2B). If the FP-biotin had been attached to Tyr 140, the masses would have been a2 = 249, b2 = 277, $a1^{+2}$ = 68, and $a2^{+2}$ = 125 amu. Peaks at 226.3, 312.4, and 329.3 are fragments of FP-biotin. Masses at 708.2 and 691.3 amu for FP-

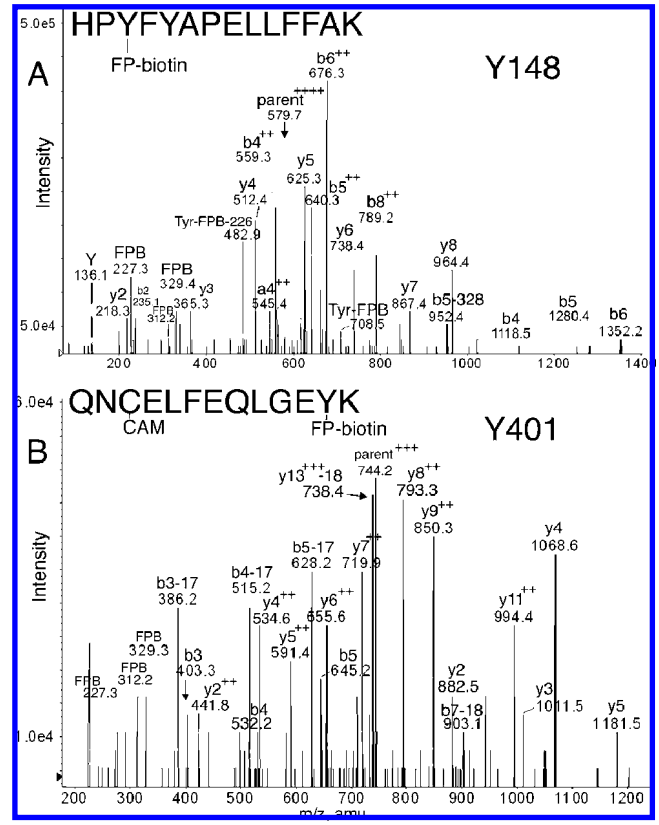

Figure 3. MS/MS spectra of albumin peptides labeled with FP-biotin on Tyrosine. (A) Tyr 148 in peptide HPYFYAPELLFFAK is labeled with FP-biotin. The quadruply charged parent ion has a mass of 579.7 *m*/*z*. The FP-biotin tyrosine immonium ion is at 708.5; after neutral loss of 226, its mass is 482.9. (B) Tyr 401 in peptide QNCELFEQLGEYK is covalently modified by FP-biotin. The triply charged parent ion in B has a mass of 744.2 *m*/*z*.

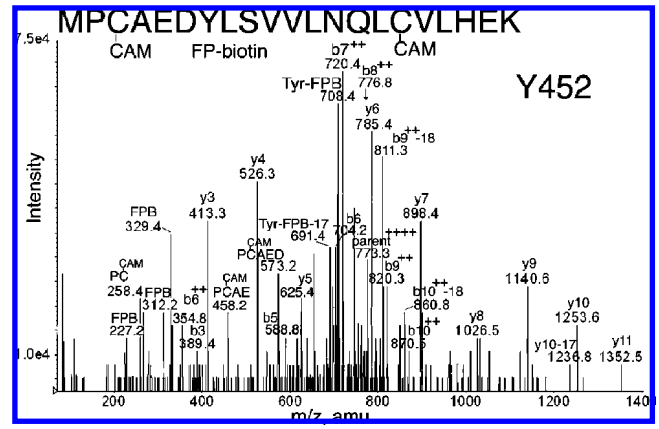

Figure 4. MS/MS spectrum of albumin peptide labeled with FP-biotin on tyrosine 452. The quadruply charged parent ion has a mass of 773.3 *m*/*z*. The carbamidomethylated cysteine is indicated as CAM. Internal fragmentation at proline yielded the 458.2 ion for PC(CAM)AE and the 573.2 ion for PC(CAM)AED. The characteristic fragments of FP-biotin at 227.2, 312.2, and 329.4 are present. The FP-biotin-tyrosine immonium ion is at 708.4 amu. After loss of an amine, the FP-biotin tyrosine immonium ion has a mass of 691.4 amu.

biotinylated tyrosine confirm the presence of FP-biotinylated tyrosine in the peptide. Analysis of the missed cleavage peptide KYLYEIAR supports labeling on Tyr 138 (data not shown).

Peptide HPYFYAPELLFFAK in Figure 3A also has two tyrosines. Evidence for labeling on Tyr 148 rather than on Tyr 150 is the presence of the b4 ion at 1118.5 amu, the $a4^{+2}$ ion at 545.4 amu, and the $b4^{+2}$ ion at 559.3 *m*/*z*. The total mass of the b4 fragment (1117.6 amu) is equal to the fragment HPYF

(545 amu) plus the added mass from FP-biotin (572 amu). Of the four residues in the b4 fragment, Tyr 148 is the most reasonable candidate for labeling. Fragment masses for b5 and b6 also support labeling of Tyr 148 rather than Tyr 150. An extensive y-ion series (y2−y8) supports the assignment of this peptide. Masses at 227.3, 312.2, and 329.4 indicate the presence of FP-biotin. Masses at 691.2 and 708.5 amu indicate the presence of FP-biotinylated tyrosine. A similar analysis was made for peptides RHPYFYAPELLFFAK and HPYFYAPELLFFAKR, which differ from HPYFYAPELLFFAK by virtue of missed cleavages (data not shown).

Peptide QNCELFEQLGEYK in Figure 3B is FP-biotinylated on Tyr 401 as demonstrated by the y2 ion at 882.5 amu, the y3 ion at 1011.5 amu, the y4 ion at 1068.5 amu, and the y5 ion at 1181.8 amu. The y2 mass is equal to the sum of Lys (147 amu), Tyr (163 amu), and the added mass of FP-biotin (572 amu). A variety of larger, multiply charged y-ion fragments support the labeling assignment. Prominent b-ion fragments confirm the identity of the peptide. Fragments at 227.2, 312.2, and 329.3 amu indicate the presence of FP-biotin in the peptide.

Peptide MPCAEDYLSVVLNQLCVLHEK in Figure 4 is FP-biotinylated on Tyr 452. The best evidence in support of this interpretation is the doubly charged mass at 720.4 *m*/*z*, which is consistent with the b7 ion plus the added mass from FP-biotin. The b7 ion consists of MPCAEDY. Of these residues, only Tyr 452 is a reasonable candidate for FP-biotinylation. The $b8^{+2}$, $b9^{+2}$, and $b10^{+2}$ ions support this interpretation. The y-series (y3−y11) supports identification of this peptide. Masses at 227.2, 312.2, and 329.4 indicate the presence of FP-biotin. Masses at 691.4 and 708.4 amu indicate the presence of FP-biotinylated tyrosine in this peptide. A missed cleavage form of this peptide, RMPCAEDYLSVVLNQLCVLHEK, was also analyzed, and the results support labeling of Tyr 452 (data not shown).

Peptide SHCIAEVENDEMPADLPSLAADFVESK in Figure 5A is FP-biotinylated on Ser 287. Existence of an FP-biotinylated serine is indicated by the major peak at 591.4 amu. This is a characteristic mass from FP-biotin that appears as the result of β-type elimination of FP-biotin from a serine adduct (Figure 5B), during collision-induced dissociation in the mass spectrometer (*12*). The complementary peptide fragment arising from this fragmentation contains a dehydroalanine in place of serine. The masses of a b-series (Δb5−Δb12) containing a dehydroalanine residue support the elimination of FP-biotin from serine. Of the residues in the b5 fragment (SHCIA), serine at position 287 is a candidate for FP-biotinylation. The cysteine might have been considered a target for labeling, but the overall mass of the fragment is consistent with carbamidomethylation on the cysteine. A y-ion series (y4−y15) supports the identification of the peptide. Additional support for the presence of FP-biotin in the peptide comes from characteristic masses at 312.1 and 329.2 amu. The absence of the characteristic mass at 227 amu is common for FP-biotinylated serine.

The MS/MS spectrum for peptide AEFAEVSK labeled by FP-biotin on Ser 232 is in Figure 6. The b- and y-ion masses support the assigned sequence. Peaks not assigned by Protein Pilot included six dehydroalanine fragments as well as the 591 amu ion of FP-biotin and the 227, 312, and 329 amu fragments of FP-biotin. These additional peaks strongly support the conclusion that Ser 232 of albumin was labeled by FP-biotin. This labeled peptide was detected by the sensitive QSTAR elite mass spectrometer but not by the QTRAP 2000 mass spectrometer. No FP-biotinylated peptides were found in the control plasma that had not been treated with FP-biotin.

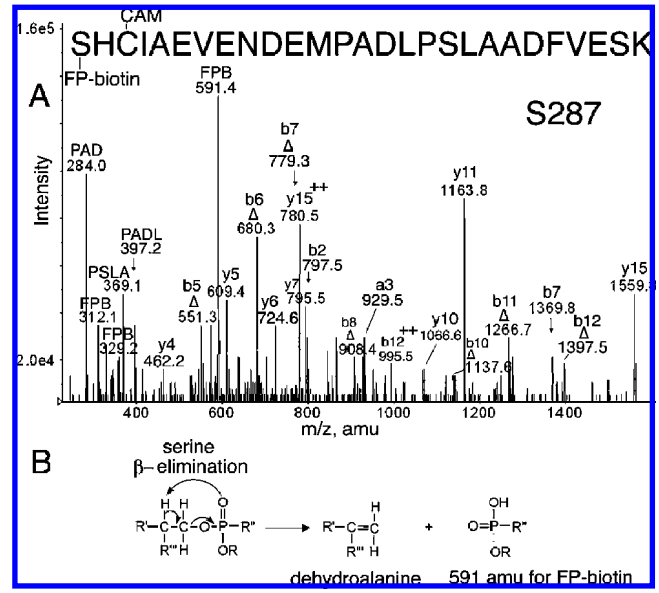

Figure 5. (A) MS/MS spectrum of albumin peptide labeled with FP-biotin on Ser 287. The triply charged parent ion has a mass of 1183.8 *m*/*z*. The carbamidomethylated (CAM) peptide carried the FP-biotin on Ser 287. The evidence for modification on serine is the presence of a b-ion series for the dehydroalanine form of the peptide, designated Δ. The 591.4 amu ion is FP-biotin released from serine where the fluoride ion has been replaced by a hydroxyl group. Release of the entire OP accompanied by formation of dehydroalanine is a characteristic of OP bound to serine. Internal fragmentation at proline yielded masses at 284.0 for PAD, 369.1 for PSLA, and 397.2 for PADL. (B) Scheme for β-elimination of the OP label from serine. Fragmentation in the mass spectrometer eliminates the OP from serine and simultaneously converts serine to dehydroalanine.

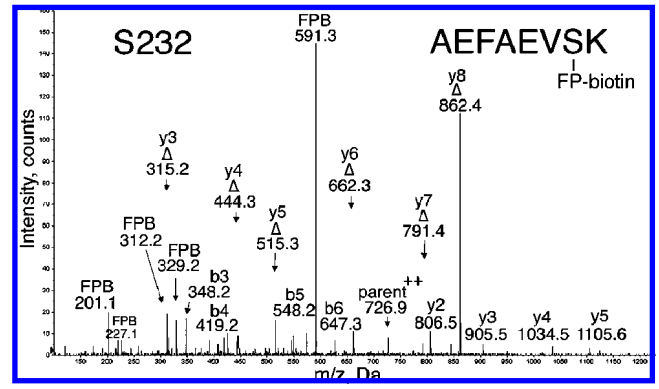

Figure 6. MS/MS spectrum of albumin peptide labeled with FP-biotin on Ser 232. This spectrum was acquired on the QSTAR elite mass spectrometer. The doubly charged parent ion is at 726.9 amu. The peak at 591.3 is FP-biotin released from serine, carrying a hydroxyl group in place of fluorine. Four y-ions (y2, y3, y4, and y5) carry FP-biotin on serine, whereas six y-ions (Δy3−Δy8) have lost FP-biotin as well as a molecule of water, thus converting serine to dehydroalanine.

Search for Other FP-Biotin-Labeled Proteins in Human Plasma. The present method identified 7 FP-biotin-labeled albumin peptides but no FP-biotin-labeled peptides from any other protein. A Western blot hybridized with Streptavidin Alexafluor-680 showed many FP-biotinylated bands in human plasma treated with FP-biotin under our conditions (data not shown). One such protein is FP-biotinylated plasma butyrylcholinesterase (*1, 14*). However, the FP-biotinylated butyrylcholinesterase peptide was not found with the present methods. FP-biotinylated peptides from proteins other than albumin are difficult to detect in the presence of the overwhelmingly high concentration of albumin. Even after depletion of albumin with Cibacron Blue, the concentration of albumin was still too high

Table 2. CPO-Labeled Human Albumin Peptides[a]

start−end	M_r	sequence	CPO-labeled
139−144	1234.6	K(CONH$_2$)Y*LYEIAR	Y138
146−159	1877.9	HPYFY*APELLFFAK	Y150
161−174	1797.8	Y*KAAFTECCQAADK	Y161
390−402	1791.8	QNCELFEQLGEY*K	Y401
411−413	547.2	Y*TK	Y411
446−466	2653.2	MPCAEDY*LSVVLNQLCVLHEK	Y452

[a] Peptide KYLYEIAR was carbamylated on the N-terminal Lys by degradation products in urea, adding a mass of 43. Chorpyrifos oxon adds a mass of 136 to the labeled tyrosine.

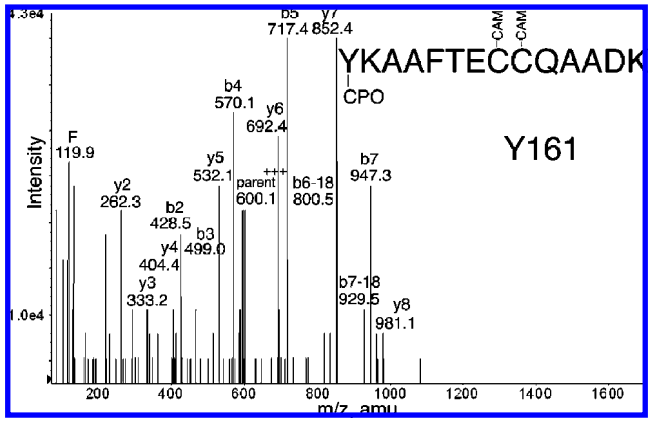

Figure 7. MS/MS spectrum of albumin peptide labeled with CPO on Tyr 161. The b2 and b3 ions support labeling on tyrosine.

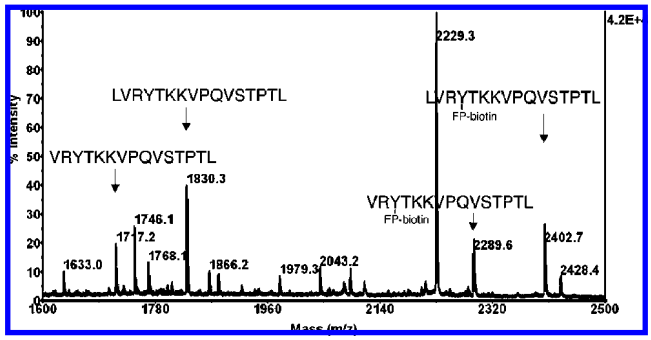

Figure 8. MALDI-TOF spectrum of pepsin-digested albumin to show labeling of Tyr 411 by FP-biotin. Pepsin digestion of albumin yields two unlabeled peptides at 1717.2 (VRYTKKVPQVSTPTL) and 1830.3 (LVRYTKKVPQVSTPTL) amu, both containing Tyr 411. Both peptides have a mass shift of 572.3 after reaction with FP-biotin, yielding the peaks at 2289.6 and 2402.7 amu. The FP-biotin is covalently bound to Tyr 411. About 50% of the Tyr 411 is labeled as calculated from cluster areas of the labeled and unlabeled peaks.

to allow detection of other FP-biotinylated peptides. Human plasma contains 5 mg of butyrylcholinesterase and 50000 mg albumin per L. In experiments not described in this report, we found OP-labeled butyrylcholinesterase in human plasma only after the butyrylcholinesterase had been purified by binding to procainamide affinity gel, thus eliminating more than 95% of the albumin.

Albumin Residues Labeled by CPO. Prolonged labeling of pure human albumin with CPO resulted in labeling of six tyrosines: Y138, Y150, Y161, Y401, Y411, and Y452 (Table 2). Four of these sites were also labeled by FP-biotin (Y138, Y401, Y411, and Y452). The HPYFYAPELLFFAK peptide was labeled on Tyr 150 by CPO, whereas it was labeled on Tyr 148 by FP-biotin. A new peptide YKAAFTECCQAADK was labeled by CPO (Figure 7) and not by FP-biotin. Labeling on tyrosine is supported by the b ion series. The identity of the peptide is supported by the y2−y8 ions. Additional MS/MS spectra for CPO-labeled peptides are in the Supporting Information (Figures S1−S5).

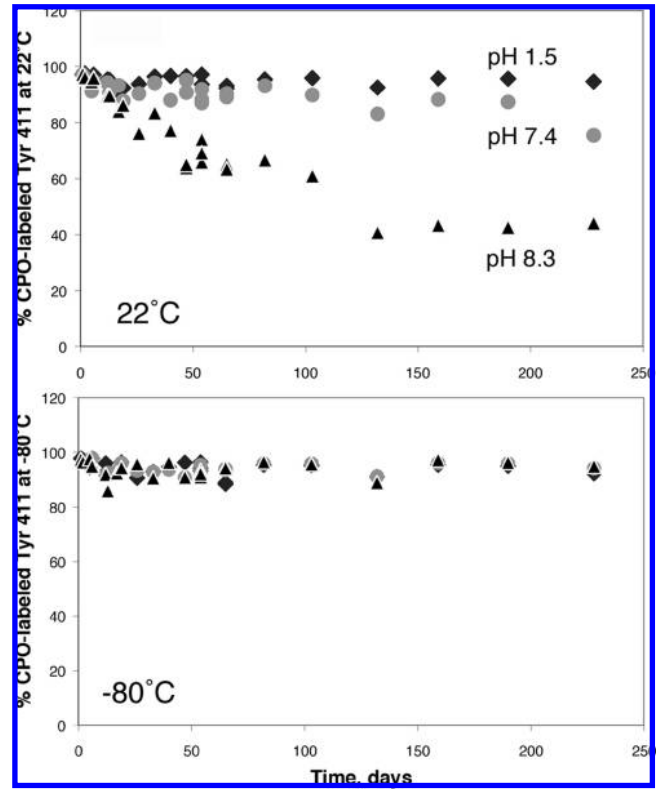

Figure 9. Stability of the diethoxyphosphate adduct of human albumin on Tyr 411. Albumin was treated with CPO to achieve 97% labeling of Tyr 411. Excess CPO was removed by dialysis. The pH of the dialyzed albumin was adjusted to 1.5, 7.4, and 8.3. CPO-albumin samples were stored at 22 and −80 °C. After various times of storage, samples were digested with pepsin and % labeling of Tyr 411 was calculated from cluster areas of labeled and unlabeled peptides in the MALDI-TOF mass spectrometer. CPO-labeled Tyr 411 was stable at pH 1.5 and 7.4 when CPO-albumin was stored at 22 °C (top panel), and it was stable at all pH values when CPO-albumin was stored at −80 °C (bottom panel). Storage of CPO-albumin at pH 8.3 at 22 °C resulted in release of half of the diethoxyphosphate group from Tyr 411 after 3.6 months.

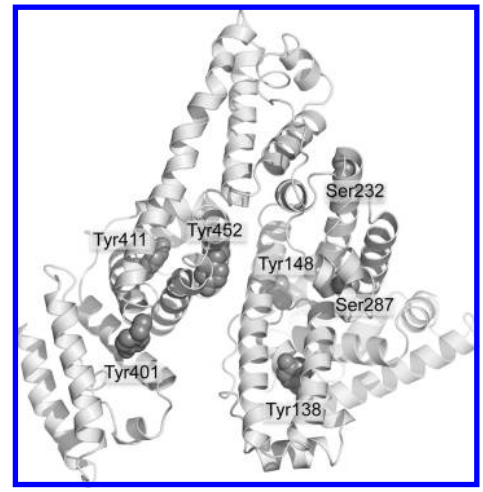

Figure 10. Crystal structure of human albumin showing surface location of Tyr 138, Tyr 148, Tyr 401, Tyr 411, Tyr 452, Ser 232, and Ser 287. The residues are shown as space-filled structures. The picture was made with PyMol software using the structure in PDB code 1bm0 (*28*).

Tyr 411 Reacts Most Readily with OP. The finding that seven tyrosines and two serines make a covalent bond with OP led to the question of which amino acid reacts most readily

with OP. To answer this question, we duplicated the conditions reported to label one molar equivalent of human albumin with DFP (*10*). MALDI-TOF analysis of pepsin-digested, DFP-treated human albumin suggested that 80% of Tyr 411 was labeled with DFP. MS/MS analysis of a tryptic digest of carbamidomethylated DFP-treated albumin confirmed that Tyr 411 in peptide Y*TK was labeled. In addition, less than 10% of peptide EFNAETFTFHADICT*LS*EK was labeled (on residues T515 and S517).

Albumin treated with FP-biotin for 2 h and digested with pepsin had 52% of its Tyr 411 labeled in peptide VRY*TKKVPQVSTPTL as calculated by MALDI-TOF mass spectrometry (Figure 8). The carbamidomethylated, trypsin-digested preparation analyzed by LC/MS/MS confirmed that Tyr 411 in peptide Y*TK was labeled with FP-biotin. Peptide HPY*FYAPELLFFAK was labeled on Tyr 148 with FP-biotin but to less than 10%. A third method to identify FP-biotinylated peptides was purification on monomeric avidin beads followed by MALDI-TOF-TOF analysis. This method yielded only one FP-biotinylated peptide, the Y*TK peptide labeled on Tyr 411.

Soman-treated albumin (150 μM soman for 2 h) analyzed by MALDI-TOF and LC/MS/MS yielded only one labeled peptide. The soman was on Tyr 411.

Albumin treated with CPO for 2 h before digestion with pepsin or trypsin and analyzed by MALDI-TOF and LC/MS/MS was labeled on Tyr 411. Approximately 30% of the Tyr 411 sites were labeled in peptides VRY*TKKVPQVSTPTL and LVRY*TKKVPQVSTPTL. In addition, less than 5% of Thr 566 in peptide ET*CFAEEGKK and less than 5% of Thr 236 and Thr 239 in peptide LVT*DLT*KVHTECCHGDLLECADDR were labeled. We conclude that Tyr 411 is the most OP reactive residue in human albumin.

Support for the conclusion that Tyr 411 is the most OP reactive residue in albumin comes from ref *2*. Williams incubated the albumin fraction of human plasma with radiolabeled sarin, digested with trypsin, purified the radiolabeled peptides by HPLC, and analyzed by LC/MS/MS. A single radiolabeled peptide was isolated. Its sequence was YTK with the isopropyl methylphosphonyl group on tyrosine.

Unstable OP-Ser and OP-Thr but Stable OP-Tyr. It was noted that serine and threonine residues were labeled in addition to tyrosine when samples had been incubated at pH 8.0−8.3 for 2−48 h but were not found in samples incubated at pH 8.3 for a month. In contrast, OP-labeled tyrosines were found even after 1 month of incubation at pH 8.3. Our stability study of CPO-labeled albumin confirmed that the Tyr 411 adduct was stable (Figure 9). Incubation at pH 7.4 and 22 °C resulted in almost no loss of the CPO label on Tyr 411 after 7 months. In contrast, about half of the label was lost after 3.6 months at pH 8.3 and 22 °C. The CPO-labeled Tyr 411 was stable at pH 1.5 and 22 °C and was stable at all pH values when the labeled albumin was stored at −80 °C. These results suggest that OP-labeled serine and threonine adducts are unstable as compared to OP-labeled tyrosine.

Surface Location of OP Reactive Residues. The crystal structure in Figure 10 shows the five tyrosines and two serines that become labeled by FP-biotin. These residues are located on the surface of the albumin molecule where they are available for reaction with OP.

Human albumin has 18 tyrosines and 24 serines but only five tyrosines and two serines made a covalent bond with FP-biotin. Their special reactivity may be explained by a nearby arginine or lysine that stabilizes the ionized hydroxyl of tyrosine or serine.

Discussion

Many OP-Reactive Residues in Human Albumin. Although Tyr 411 is the most OP reactive residue in human albumin, an additional eight amino acids were labeled when the OP concentration was high and the reaction time was prolonged. The reaction with FP-biotin at pH 8.0 resulted in the labeling of five tyrosines and two serines in albumin. CPO labeled six tyrosines (two of which were different from those labeled by FP-biotin) and no serines. We agree with Means and Wu (*10*) that about 90% of the label is on Tyr 411 and 10% is on other residues.

The pK_a of the tyrosine hydroxyl group is 10.1 and of the serine hydroxyl group is approximately 16, based on comparison to ethanol (*15*). In the absence of special activation, less than 1% of the tyrosines and less than 0.000001% of the serines would be expected to be ionized at pH 8.0. Ionized forms react preferentially with OP, so the reactivity of tyrosine and especially of serine with OP would be expected to be poor at pH 8. The special reactivity of Tyr 411 suggests that the pK_a of this particular tyrosine has been lowered. Means and Wu identified an OP reactive residue in albumin that had a pK_a of 8.3 (*10*). It is likely that Tyr 411 corresponds to that residue.

Albumin as an OP Scavenger. Our results show that albumin is an OP scavenger, undergoing a covalent reaction with OP. As such, albumin contributes to detoxication of OP. A significant amount of OP can be bound by albumin because the concentration of albumin in serum is high (≈0.6 mM), even though the rate of reaction with OP is slow (*3*).

Tyrosines with an abnormally low pK_a are involved directly or indirectly in the catalytic activity of numerous enzymes including glutathione S-transferase (*16*), asparaginase (*17*), β-lactamases (*18*), and old yellow enzyme (*19*). Lowering the pK_a of tyrosines in albumin by modifying their environment, either by mutagenesis or by chemical modification of vicinal residues, would increase the reactivity of albumin with OP. Specific nitration of tyrosine by tetranitromethane was found to lower the pK_a of tyrosine to 6.8 (*20*). Another nitration reagent of tyrosine, peroxynitrite, was found to increase the catalytic activity of a few enzymes (*21*). Thus, specific nitration of tyrosine residues in albumin could also lead to a gain in reactivity of this protein, increasing its scavenging properties.

No Aging of OP-Tyrosine Adducts. When soman or DFP are bound to acetylcholinesterase or butyrylcholinesterase, the OP loses an alkyl group in a process called aging (*22*−*25*). An aged soman-labeled peptide would have an added mass of 78 rather than 162; an aged DFP-labeled peptide would have an added mass of 122 rather than 164; an aged CPO-labeled peptide would have an added mass of 108 rather than 136. Masses corresponding to aged OP-labeled peptides were not found in MS scans. We conclude that albumin OP adducts on tyrosine do not age.

Support for this conclusion comes from the work of others (*2, 26*). Human albumin covalently labeled with soman or sarin and treated with sodium fluoride to release the OP yielded intact soman and sarin. Soman-tyrosine adducts isolated from nerve agent-treated guinea pigs contained the pinacolyl group of soman.

The absence of aging is a special advantage for OP-albumin as a biomarker because it allows for a more precise identification of the OP. In contrast, soman and sarin exposure cannot be distinguished when the biomarker is cholinesterase, where aging of OP adducts occurs rapidly.

OP Labeling of Albumin in Living Animals. Guinea pigs treated with the nerve agents soman, sarin, cyclosarin, or tabun

have nerve agent-labeled albumin in their blood (*2*). The OPs are bound to tyrosine. The tabun-tyrosine and soman-tyrosine adducts were detected in blood 7 days postexposure, indicating that the adducts are stable. The adducts had not undergone aging and had not been released from tyrosine by treatment of the guinea pigs with oxime. These are characteristic features of OP adducts on albumin. Mice treated with a nontoxic dose of FP-biotin by intraperitoneal injection had FP-biotinylated albumin in blood and muscle (*1*). These examples show that OP binds covalently to albumin under physiological conditions and that OP-albumin adducts could therefore be useful as biomarkers of OP exposure (*27*). Low OP doses make a covalent bond with Tyr 411 of albumin.

Significance. Our results suggest that OP exposure could be monitored by mass spectrometry of OP-albumin adducts or with antibodies against OP-albumin adducts. The surface location of the OP-binding sites in albumin suggests that these epitopes may be available for reaction with antibodies. This is in distinct contrast with acetylcholinesterase and butyrylcholinesterase where the OP binding site is buried deep within the molecule, making it unavailable to antibodies. Antibodies to OP-albumin would be primarily against OP-labeled Tyr 411 because Tyr 411 is the most reactive residue at low OP concentrations. The common OP pesticides yield either diethoxyphosphate or dimethoxyphosphate adducts. Therefore, only two antibodies would be needed for detection of exposure to common OP pesticides. The studies described here support investigation into whether albumin could be engineered to become a more efficient OP scavenger.

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Supporting Information Available: MS/MS spectra for CPO-labeled peptides (Figures S1−S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Tyrosines of Human and Mouse Transferrin Covalently Labeled by Organophosphorus Agents: A New Motif for Binding to Proteins that Have No Active Site Serine

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The expectation from the literature is that organophosphorus (OP) agents bind to proteins that have an active site serine. However, transferrin, a protein with no active site serine, was covalently modified *in vitro* by 0.5mM 10-fluoroethoxyphosphinyl-N-biotinamido pentyldecanamide, chlorpyrifos oxon, diisopropylfluorophosphate, dichlorvos, sarin, and soman. The site of covalent attachment was identified by analyzing tryptic peptides in the mass spectrometer. Tyr 238 and Tyr 574 in human transferrin and Tyr 238, Tyr 319, Tyr 429, Tyr 491, and Tyr 518 in mouse transferrin were labeled by OP. Tyrosine in the small synthetic peptide ArgTyrThrArg made a covalent bond with diisopropylfluorophosphate, chlorpyrifos oxon, and dichlorvos at pH 8.3. These results, together with our previous demonstration that albumin and tubulin bind OP on tyrosine, lead to the conclusion that OP bind covalently to tyrosine, and that OP binding to tyrosine is a new OP-binding residue. The OP-reactive tyrosines are activated by interaction with Arg or Lys. It is suggested that many proteins in addition to those already identified may be modified by OP on tyrosine. The extent to which tyrosine modification by OP can occur *in vivo* and the toxicological implications of such modifications require further investigation.

Key Words: **plasma; soman; sarin; mass spectrometry; tyrosine residue; transferrin.**

We have previously shown that many proteins in plasma react with the organophosphorus agent (OP) FP-biotin (10-fluoroethoxyphosphinyl-N-biotinamido pentyldecanamide) (Peeples *et al*., 2005). Our goal is to identify these proteins. The proteins that react most rapidly with FP-biotin and with other OP are enzymes in the serine hydrolase family, for example, butyrylcholinesterase, acetylcholinesterase, acylpeptide hydrolase, fatty acid amide hydrolase, arylformamidase, and neuropathy target esterase-lysophospholipase (Casida and Quistad, 2004; Richards *et al*., 2000). The residue that is labeled in these enzymes is the active site serine in the consensus sequence GlyXSerXGly. However, proteins with no consensus active site serine constitute another group of OP-reactive proteins, where OP bind to tyrosine. Papain and bromelain bind diisopropylfluorophosphate (DFP) on tyrosine (Chaiken and Smith, 1969; Murachi *et al*., 1965). Mass spectrometry has allowed identification of OP-binding to tyrosines in albumin and tubulin (Ding *et al*., 2008; Grigoryan *et al*., 2008; Li *et al*., 2007, 2008). The present report adds transferrin to this list. Analysis of the reactive peptides from these proteins shows that a new motif of OP binding has emerged.

Our strategy uses FP-biotin, initially, to label the OP-reactive proteins in plasma. Through the use of the fluorescent probe Streptavidin-Alexa 680 and the biotin tag, the proteins that are OP-reactive in serum can be visualized. The biotin tag also provides a means for purification of the labeled proteins and peptides by binding to avidin-agarose beads. The FP-biotin–labeled proteins and peptides are identified by mass spectrometry. It is relatively simple to identify a protein by mass spectrometry because only a few peptides from a protein are needed for a positive identification. However, it is often difficult to find a specific labeled peptide. Convincing proof that a protein is OP-labeled comes from identifying the labeled peptide and the amino acid in that peptide that is modified by OP. To provide this proof we label pure protein with OP, digest with trypsin, separate and enrich the OP-labeled peptides by reverse phase high-performance liqid chromatography (HPLC), and determine the peptide sequence and site of attachment by collision-induced dissociation in the mass spectrometer.

Not all OP are expected to bind covalently to a particular protein. For example, the positively charged echothiophate and VX react rapidly with acetylcholinesterase, but only poorly with carboxylesterase (Maxwell and Brecht, 2001). Therefore we treated human and mouse transferrin with six different OP. We found human transferrin peptides labeled with six OP, and mouse transferrin peptides labeled with five OP.

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The common feature in the motif for OP-reactive tyrosines is the presence of a positively charged arginine or lysine within five residues of the tyrosine. This OP-binding motif was tested with two synthetic peptides, both containing tyrosine, but only one containing nearby arginines. Tyrosine formed a covalent bond with OP in both peptides, but the reaction proceeded more readily with ArgTyrThrArg than with SerTyrSerMet. Additional work with small peptides needs to be done to determine the optimum peptide sequence of the OP reactive peptide and to determine its rate of reaction with OP.

EXPERIMENTAL PROCEDURES

Materials. FP-biotin (Mr 592.32) was custom synthesized in the laboratory of Dr Charles Thompson at the University of Montana, Missoula, MT. A 2mM FP-biotin solution in dimethyl sulfoxide was stored at −80°C. Chlorpyrifos oxon (ChemService, Inc. West Chester, PA; MET-674B) was dissolved in ethanol and stored at −80°C. Dichlorvos (ChemService Inc.; PS-89) was dissolved in methanol. Soman and sarin from CEB (Vert-le-Petit, France) were dissolved in isopropanol. Immun-Blot polyvinylidene difluoride (PVDF) membrane for protein blotting, 0.2 μm (162-0177) and Affi-gel blue (153-7301), a cross-linked agarose bead with covalently attached Cibacron Blue F3GA dye, were from Bio-Rad Laboratories, Hercules, CA. Streptavidin Alexa Fluor 680 (S-21378) was from Molecular Probes, Eugene, OR. Porcine trypsin (Promega, Madison, WI; V5113 sequencing grade modified trypsin) at a concentration of 0.4 μg/μl in 50mM acetic acid was stored at −80°C. Immobilized tetrameric avidin-agarose beads (Pierce 20219) and immobilized monomeric avidin-agarose beads (Pierce 20228) were used to purify FP-biotinylated proteins as well as FP-biotinylated peptides. Human butyrylcholinesterase was purified from outdated human plasma as described (Lockridge *et al*., 2005) and labeled with FP-biotin as described (Peeples *et al*., 2005). Mouse plasma was from strain 129Sv mice. Human holo-transferrin (T0665), human apo-transferrin (T4382), mouse apo-transferrin (T0523), bovine holo-transferrin (T1408), human alpha 2-macroglobulin (M6159), human alpha 1-antitrypsin (A9024), human complement C3 (C2910), nitrilotriacetic acid (N9877), Coomassie blue R250 (Brillant Blue R), and diisopropylfluorophosphate (D0879, a liquid with a concentration of 5.73M) were from Sigma, St Louis, MO. ProbeQuant G50 micro column was from Amersham (27-5335-01). Peptide ArgTyrThrArg (Mr 594.7) was custom synthesized and purified to > 95% by Genscript Corp., Piscataway, NJ. Peptide SerTyrSerMet (#20621; Mr 486.5) was from AnaSpec, Inc., San Jose, CA. Alpha-cyano-4-hydroxy cinnamic acid, Glu Fibrinopeptide B, and Cal Mix 1 were from Applied Biosystems (MDS Sciex, Foster City, CA).

Blot of FP-biotin–labeled proteins from a nondenaturing polyacrylamide electrophoresis gel. Two hundred microliters of human or mouse plasma was treated with 100μM FP-biotin for 24 h at 37°C. Ten- to 30-μl aliquots were subjected to gel electrophoresis on nondenaturing polyacrylamide gradient gels, 4–22.5% (wt/vol), cast in a Hoefer gel apparatus. The polyacrylamide gel electrophoresis (PAGE) gels were 0.75 mm thick, 15 cm wide, and 12 cm high. Nondenaturing gels were used because albumin separates from other plasma proteins on a nondenaturing gel, but not on a sodium dodecyl sulfate (SDS) gel. Proteins were electrophoretically transferred to a PVDF membrane using a Bio-Rad Trans-blot apparatus. The blot was hybridized with a fluorescent probe, Streptavidin Alexa Fluor 680, as described (Peeples *et al*., 2005). Fluorescence intensity was captured in the Odyssey Infrared Imaging System (LiCor).

Blot of FP-biotin–labeled proteins from an SDS PAGE gel. Purified bovine holo-transferrin (76–81 kDa), human alpha 2-macroglobulin (tetramer 720 kDa; monomer 179 kDa), human alpha 1-antitrypsin (52 kDa), and human complement C3 (115 and 70 kDa) were dissolved in phosphate buffered saline to a concentration of 500 pmol in 50 μl. Proteins were treated with 100μM FP-biotin at 37°C for 20 h. Control samples were treated with dimethyl sulfoxide but no FP-biotin. Unreacted FP-biotin was removed by passing the samples through a G50 spin column. Samples were boiled in the presence of dithiothreitol and SDS and loaded on a 4–22.5% (wt/vol) polyacrylamide gradient SDS gel. After electrophoresis, some gels were stained for protein with Coomassie blue R250. For other gels, the protein was transferred to a PVDF membrane where the biotin tag was detected with Streptavidin Alexa Fluor 680, as described (Peeples *et al*., 2005). The amount of protein loaded on the Coomassie stained gel ranged from 3.5 to 70 μg, and that on the gel used for transfer to PVDF ranged from 1.5 to 30 μg.

Isolation of FP-biotin–labeled proteins with avidin-agarose beads. Plasma was depleted of albumin by chromatography on Affi-gel blue because albumin reacts extensively with FP-biotin and can deplete the FP-biotin pool making reaction with other proteins more difficult. The albumin-depleted plasma was treated with 100μM FP-biotin for 24 h at 37°C, and dialyzed to remove excess FP-biotin. The FP-biotinylated proteins were purified by binding to tetrameric avidin-agarose beads and separated by SDS gel electrophoresis as described (Peeples *et al*., 2005).

Purification of FP-biotin–labeled peptides. In some experiments the FP-biotin–labeled peptides rather than FP-biotin–labeled proteins were purified by binding to monomeric avidin-agarose beads. After washing with 1M NaCl in pH 8.0 buffer, followed by water washes to desalt the column, the peptides were released with 10% (vol/vol) acetic acid.

Reaction of pure transferrin with OP. A 1 mg/ml solution of human or mouse transferrin in 0.1M TrisCl pH 8.5, or in phosphate buffered saline, or in 10mM TrisCl pH 8.5, 0.01% (wt/vol) sodium azide was treated with 0.5mM OP at 37°C for 16 h. The accession numbers in the SwissProt database are

P02787 for human and Q921I1 for mouse transferrin. Their molecular weight is about 75,000 after the 19 amino acid signal peptide is subtracted.

Tryptic peptides for mass spectrometry. Three protocols were used to prepare peptides for mass spectrometry. (1) Proteins in SDS PAGE gel slices were digested with trypsin. The peptides were extracted from the gel and analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) as described (Peeples *et al.*, 2005). This protocol was used only for the preliminary experiments where the data are not shown. (2) Pure proteins in solution were denatured in 8M urea, reduced with 10mM dithiothreitol at pH 8, carbamidomethylated (CAM) with 50mM iodoacetamide, and desalted by dialysis against 10mM ammonium bicarbonate. The CAM proteins were digested with trypsin at a ratio of 50:1 (w/w) at 37°C for 16 h. Peptides were separated offline by reverse phase HPLC on a 100 × 4.60 mm Phenomenex C18 column eluted with a 60 min gradient from 0 to 60% (vol/vol) acetonitrile versus 0.1% (vol/vol) trifluoroacetic acid at a flow rate of 1 ml/min. Fractions were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry to locate the OP-labeled peptides. Selected samples were dried, dissolved in 50% acetonitrile, 0.1% formic acid, and infused into the QTRAP 4000 mass spectrometer for MS/MS analysis. Peptides were infused into the mass spectrometer when the mass of the parent ion was known. (3) Trypsin digested pure protein was analyzed a second way to allow for the possibility that the mass of the parent ion was unknown. The offline HPLC step was omitted, and peptides were separated on a nanocolumn whose output was electrosprayed into the QTRAP 2000 mass spectrometer. MS/MS spectra were acquired for three peptides every 2.8 s.

Reaction of model peptides with OP. Peptides ArgTyrThrArg (0.17mM) and SerTyrSerMet (0.17mM) in 10mM ammonium bicarbonate pH 8.3 were treated with 0.1, 0.2, or 1mM DFP, chlorpyrifos oxon (CPO), or dichlorvos for 16 h at 37°C. Aliquots were spotted on a MALDI target plate, overlaid with CHCA (α-cyano-4-hydroxy cinnamic acid) matrix, and analyzed by MALDI-TOF as well as by MALDI-TOF-TOF mass spectrometry and by LC/MS/MS on the QTRAP 2000.

Percent labeling. The percent labeled peptide in a given MALDI-TOF spectrum was calculated by dividing the cluster area of the labeled peptide by the sum of the cluster areas for the unlabeled and labeled peaks in a given spectrum. The assumption in this calculation is that the labeled and unlabeled peptides ionize equally well. In a previous publication (Lockridge *et al.*, 2008) we checked the validity of our MALDI-TOF quantitation method by using a second method to quantitate labeled and unlabeled peptides. The second method was amino acid composition analysis. Both methods showed that 27% of a particular peptide was labeled, thus validating the MALDI-TOF quantitation method.

MALDI-TOF mass spectrometry. All peptide samples were screened by MALDI-TOF before they were analyzed in the QTRAP mass spectrometer because MALDI-TOF is a quick way to obtain peptide masses and thereby a suggestion of whether an OP-labeled peptide is present. Salt-free peptides were spotted on a target plate in 0.5-μl aliquots, allowed to dry, and overlaid with 10 mg/ml CHCA matrix in 50% acetonitrile, 0.1% trifluoroacetic acid. Mass spectra were acquired with the MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA). Each scan is the sum of 500 laser shots—50 shots were taken at one location on the spot and then the laser automatically moved to a new location to avoid burning out the sample. Laser intensity was adjusted to obtain maximum signal intensity without exceeding the saturation limit. The mass spectrometer was calibrated against Cal Mix 1 (des-Arg Bradykinin, Glu Fibrinopeptide B, angiotensin I, and neurotensin).

LC/MS/MS on a tandem quadrupole mass spectrometer. Tryptic digests of OP-labeled human and mouse transferrin were analyzed by this method. A 10-μl aliquot of peptides in 5% acetonitrile, 0.1% formic acid, at a concentration of about 2 pmol/μl, was injected into the HPLC nanocolumn (#218MS3.07515 Vydac C18 polymeric rev-phase, 75 micron ID × 150 mm long; P.J. Cobert Assoc, St Louis, MO). Peptides were separated with a 90 min linear gradient from 0 to 60% acetonitrile at a flow rate of 0.3 μl/min and electrosprayed through a nanospray emitter (fused silica, 360 μm OD × 75 μm ID × 15 μm taper; New Objective) directly into the mass spectrometer. Mass spectra, high resolution mass spectra, and product ion spectra (MS/MS) were acquired on a QTRAP 2000 triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) using the trap (enhanced sensitivity) mode. An ion-spray voltage of 1900 volts was maintained between the emitter and the mass spectrometer. Information-dependent acquisition was used to acquire data for the three most intense peaks in each cycle, having a charge of +1 to +4, a mass between 200 and 1700 *m/z*, and an intensity > 10,000 counts per s. Precursor ions were excluded for 30 s after one MS/MS spectrum had been collected. The collision cell was pressurized to 40 μTorr with pure nitrogen and collision energies between 20 and 40 eV were determined automatically by the Analyst 1.4.1 software, based on the mass and charge of the precursor ion. The mass spectrometer was calibrated using fragment ions generated from collision-induced dissociation of Glu fibrinopeptide B (Sigma). The MS/MS data were processed using Analyst 1.4.1 software and submitted to Mascot for identification of peptide sequences modified by OP on Tyr, Ser, or Thr (Perkins *et al.*, 1999).

Infusion in the QTRAP 4000 mass spectrometer. HPLC purified OP-labeled human and mouse transferrin tryptic peptides were analyzed by this method when the mass of the parent ion was known from MALDI-TOF experiments. Peptides were dissolved in 50% acetonitrile, 0.1% formic acid

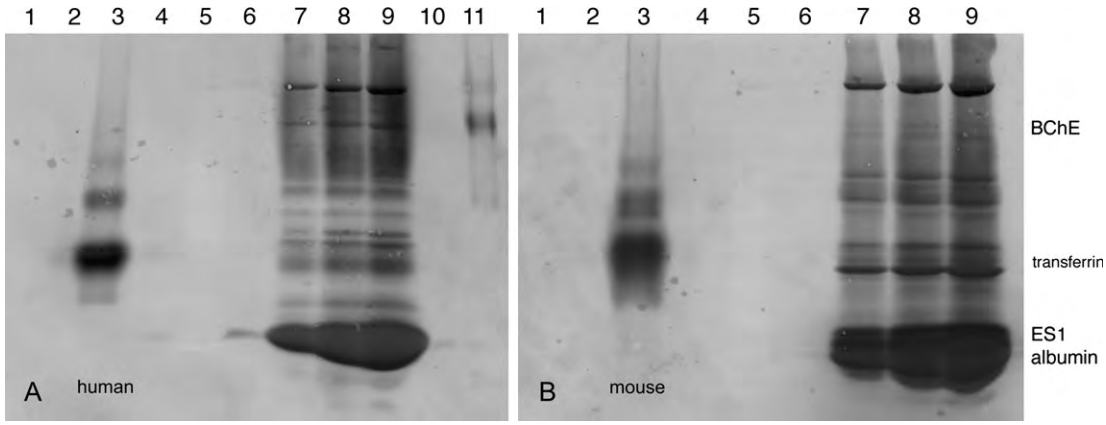

FIG. 1. Blots showing FP-biotin–reactive proteins in human (A) and mouse (B) plasma. FP-biotinylated plasma proteins were separated on nondenaturing polyacrylamide gels and transferred to PVDF membranes. Blots were hybridized with the fluorescent probe Streptavidin Alexa Fluor 680. A1, 20 μg human transferrin; A2, blank; A3, 20 μg FP-biotinylated human transferrin; A4, blank; A5, 5 μl of human plasma; A6, blank; A7, 3.3 μl of FP-biotinylated human plasma; A8, 6.6 μl of FP-biotinylated human plasma; A9, 9.9 μl of FP-biotinylated human plasma; A10, blank; A11, 1 pmol FP-biotinylated human butyrylcholinesterase (BChE). B1, 20 μg mouse transferrin; B2, blank; B3, 20 μg FP-biotinylated mouse transferrin; B4, blank; B5, 5 μl of mouse plasma; B6, blank; B7, 5 μl of FP-biotinylated mouse plasma; B8, 10 μl of FP-biotinylated mouse plasma; B9, 15 μl of FP-biotinylated mouse plasma. The heavy band in B contains mouse ES1 carboxylesterase and mouse albumin. ES1 carboxylesterase in mouse plasma does not separate well from albumin on a nondenaturing gel. Human plasma does not contain carboxylesterase.

to a concentration of 2–6 pmol/μl. Peptides were infused into the QTRAP 4000 (Applied Biosystems) mass spectrometer at a flow rate of 0.3 μl/min through an 8 μm emitter (#FS360-50-8-D, New Objective) via a 25-μl Hamilton syringe mounted on a Harvard pump. Five hundred MS/MS spectra were accumulated for each parent ion.

Binding of ferric ion to transferrin. The normal function of transferrin is iron transport through the blood. Binding of ferric ion is a measure of the functional viability. Titration of transferrin with ferric ion was performed as described (Welch and Skinner, 1989). A 1 mg/ml solution of apo-human transferrin was prepared in 0.1M TrisCl pH 8.5 containing 5mM Na_2CO_3. Half of the transferrin solution was incubated with 100μM FP-biotin at 37°C for 16 h, resulting in the labeling of Tyr 238 and Tyr 574. The other half was the unlabeled control. Protein concentration was determined with the CB-X protein assay kit (Genotech, St Louis, MO; #786-12X). FP-biotin–labeled as well as control apo-transferrin were titrated with ferric nitrilotriacetate (FENTA) to determine the stoichiometry of ferric ion binding. Each 1 ml solution of transferrin was titrated by sequential additions of 5 μl of 1mM FENTA. Binding was monitored by following the change in absorbance at 470 nm.

RESULTS

Human and Mouse Plasma Proteins that Bind FP-Biotin

The blots in Figures 1A and 1B show at least 12 bands in both human and mouse plasma that bind FP-biotin (Figs. 1A and 1B, lanes 7, 8, 9). The most intense band in human plasma is FP-biotin–labeled albumin. The most intense band in mouse plasma contains two proteins: FP-biotin–labeled carboxylesterase ES1 and FP-biotin–labeled albumin (Fig. 1B, lanes 7, 8, 9). A major difference between human and mouse plasma is that only mouse plasma contains carboxylesterase (Li *et al*., 2005). Albumin, ES1 carboxylesterase, and butyrylcholinesterase have previously been identified as proteins in mouse plasma that bind FP-biotin (Peeples *et al*., 2005).

The search for additional OP-reactive proteins in plasma led to preliminary identification of human transferrin. This preliminary identification resulted from LC/MS/MS mass spectral analysis of an in-gel, tryptic digest of a band from an SDS PAGE gel. Data were acquired on the QTRAP 2000 mass spectrometer. Proteins applied to this gel had been labeled with FP-biotin and purified with avidin-agarose (data not shown). Mass spectra identified transferrin, but not the labeled peptide.

To confirm that transferrin was actually labeled by FP-biotin, pure human and mouse transferrin treated with FP-biotin were visualized on a blot hybridized with Streptavidin Alexa Fluor 680. Lane 3 in Figures 1A and 1B shows an intense band for FP-biotinylated transferrin, thus confirming that human and mouse transferrin bind FP-biotin.

The preliminary LC/MS/MS mass spectral experiments also showed alpha-2-macroglobulin, alpha-1-antitrypsin, and complement C3 as proteins in human plasma that could be labeled with FP-biotin (data not shown). As with transferrin, the FP-biotinylated plasma proteins had been purified on avidin-agarose beads, separated by SDS gel electrophoresis, digested with trypsin, and analyzed by LC/MS/MS. The labeled peptides from these proteins were not found in these preliminary experiments. To confirm that these proteins made a covalent bond with FP-biotin, highly purified preparations of the proteins were treated with FP-biotin and subjected to SDS

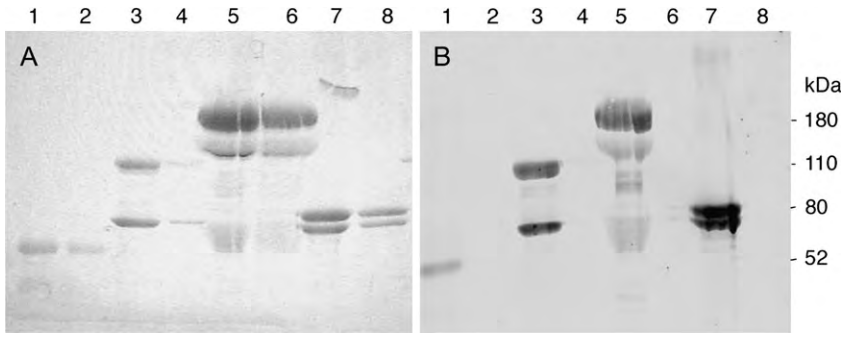

FIG. 2. Covalent binding of FP-biotin to human alpha-1-antitrypsin, human complement C3, human alpha-2-macroglobulin, and bovine holo-transferrin. (A) Coomassie stained SDS gel. (B) Blot hybridized with Streptavidin Alexa Fluor 680 to visualize FP-biotinylated proteins transferred to PVDF membrane from the gel. Lane 1, FP-biotinylated alpha-1-antrypsin; lane 2, unlabeled alpha-1-antitrypsin; lane 3, FP-biotinylated complement C3; lane 4, unlabeled complement C3; lane 5, FP-biotinylated alpha-2-macroglobulin; lane 6, unlabeled alpha-2-macroglobulin; lane 7, FP-biotinylated transferrin; lane 8, unlabeled transferrin.

gel electrophoresis. The labeled proteins were transferred from the gel to PVDF membrane. The membrane was hybridized with Streptavidin Alexa Fluor 680 (Fig. 2B). A second gel, containing higher quantities of protein, was stained with Coomassie blue R250 (Fig. 2A). Figure 2 (lanes 2, 4, 6, 8) shows that control proteins, not treated with FP-biotin, have a Coomassie stained band in panel A, but no fluorescent band in panel B. On the other hand, proteins treated with FP-biotin (lanes 1, 3, 5, 7) have a band in panel A as well as in panel B. The blot confirms that human alpha-1-antitrypsin, human complement C3, human alpha-2-macroglobulin, and bovine holo-transferrin covalently bind FP-biotin.

No further results were obtained for alpha-1-antitrypsin, complement C3, and alpha-2-macroglobulin. The site for covalent attachment of FP-biotin in these proteins is unknown.

Tyr 238 and Tyr 574 of Human Transferrin Bind OP

Structures of OP are in Figure 3. FP-biotinylated, CAM tryptic peptides of human transferrin were purified on monomeric avidin beads and their masses determined by MALDI-TOF mass spectrometry. Every peak in the MALDI-TOF spectrum was fragmented with the MALDI-TOF-TOF feature of the mass spectrometer. The MS/MS scans were examined for the presence of ions characteristic of FP-biotin at 227, 312, and 329 amu (Schopfer *et al*., 2005). A peptide that fragmented to yield these ions was labeled with FP-biotin. Additional information about the peptide was obtained in the QTRAP 4000 mass spectrometer.

Two FP-biotin–labeled peptides were identified in human transferrin. The labeled tyrosines are Tyr 238 and Tyr 574, when numbering is for the mature human transferrin protein (MacGillivray *et al*., 1982) from which the 19 amino acid signal peptide has been deleted (Swiss Prot accession #P02787). Both holo- and apo-human transferrin bound FP-biotin.

The MS/MS spectrum in Figure 4A shows that FP-biotin is covalently attached to tyrosine in peptide LysProValAspGlu-Tyr*Lys (KPVDEY*K). The parent ion mass (726 *m/z*, doubly charged) is equal to the peptide mass plus the added mass from FP-biotin (572 amu). Fragments at 227.3, 312.5, and 329.5 amu are characteristic for the presence of FP-biotin. Absence of a mass at 591 amu suggests the FP-biotin is bound to tyrosine (Schopfer *et al*., 2005), because the 591 amu fragment of FP-biotin is released from serine but not from tyrosine. The masses at 708.8 and 691.6 amu are commonly seen in the fragmentation spectrum of FP-biotinylated tyrosine and are consistent with the immonium ion of FP-biotinylated tyrosine and its deamino counterpart (unpublished observations). The y-ion series (y2–y6) is consistent with FP-biotin attached to the C-terminal peptide, TyrLys (YK). Of these two residues, tyrosine is the most likely candidate for carrying the label. Had the lysine been labeled, trypsin would not have recognized that lysine as a cleavage site. The b-ion series (b1–b5) supports the identification of the peptide.

Peptide LysProValAspGluTyr*Lys (KPVDEY*K) of human transferrin also covalently bound DFP, chorpyrifos oxon, dichlorvos, soman, and sarin on Tyr 238 as shown in Figures 4B–F. The isopropyl group of DFP and the pinacolyl group of soman were released by collision-induced dissociation in the QTRAP mass spectrometer (Figs. 4B and 4E). However, the isopropyl group was not released from sarin by matrix-assisted laser desorption ionization (Fig. 4F). The methylphosphotyrosine immonium ion at 214 amu is characteristic for soman and sarin adducts on tyrosine (Figs. 4E and 4F).

The spectrum in Figure 5A shows that FP-biotin is covalently attached to tyrosine in peptide LysProValGluGlu-Tyr*AlaAsnCysHisLeuAlaArg (KPVEEY*ANCHLAR). The parent ion mass (541.4 *m/z*, quadruply charged) is consistent with the mass of the peptide plus the added mass of FP-biotin. The characteristic fragments at 227.3, 312.6, 329.6, 708.7, and 691.6 amu along with the absence of a fragment at 591 amu are indicative of FP-biotin bound to a tyrosine in this peptide. Minor fragments at 1248.6, 1378.0, and 1507.0 amu are consistent with FP-biotinylated peptides y8–y10 that have lost 328 amu during collision-induced dissociation in the mass spectrometer. Three hundred and twenty-eight amu is the

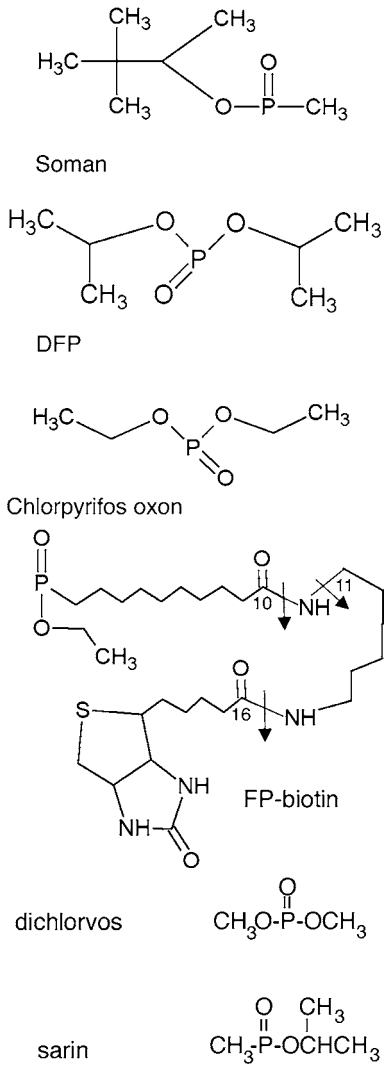

FIG. 3. Structures of the phosphoryl moieties that have become attached through phosphorus to tyrosine. Covalent binding to tyrosine results in loss of the fluoride ion from soman, DFP, FP-biotin, and sarin, of the dichlorovinyl alcohol group from dichlorvos, and of the trichloropyridinol group from chlorpyrifos oxon. Tyrosine loses one hydrogen. The added mass is 162.2 for soman, 164.1 for DFP, 136.0 for chlorpyrifos oxon, 572.3 for FP-biotin, 108.0 for dichlorvos, and 120.0 for sarin. The arrows in FP-biotin indicate fragmentation sites. A 227 amu ion is produced by cleavage between carbon 16 and the adjacent nitrogen. A 329 amu ion is produced by cleavage between carbon 10 and the adjacent nitrogen. The 312 amu ion is produced by loss of the amine from the 329 ion.

neutral counterpart of the 329 amu characteristic fragment from FP-biotin. The y-ion series (y1–y7) and the b-ion series (b1–b5) account for all the residues in the peptide except for the tyrosine which appears to carry the FP-biotin label.

Peptide LysProValGluGluTyr*AlaAsnCysHisLeuAlaArg (KPVEEY*ANCHLAR) of human transferrin also covalently bound DFP, chlorpyrifos oxon, dichlorvos, soman and sarin on Tyr 574, as shown in Figures 5B–F. The methylphosphotyrosine immonium ion at 214 amu is characteristic of soman and sarin-labeled tyrosine (Figs. 5E and 5F). Collision-induced fragmentation in the QTRAP mass spectrometer resulted in loss of the pinacolyl group from soman, and the isopropyl group from sarin (Figs. 5E and 5F), indicated by loss of 84 and 42 amu. Analyses of the spectra support the labeling of tyrosine. Details are given in the figure legends.

Mouse Transferrin Labeled on Tyr 238, Tyr 319, Tyr 429, Tyr 491, Tyr 518.

Five tyrosines in mouse transferrin were labeled by FP-biotin and chlorpyrifos oxon (Table 1). Two tyrosines were labeled by DFP, three by sarin, and two by soman. No residues were labeled by dichlorvos. Both holo- and apo- mouse transferrin bound FP-biotin (data not shown).

No Aging

Aging of OP-modified acetylcholinesterase and butyrylcholinesterase involves the loss of an alkyl group from the OP. For example, soman-inhibited acetylcholinesterase loses the pinacolyl group during aging so that the added mass for aged soman is 72 rather than 162 amu. The LC/MS/MS data sets for soman and CPO-labeled transferrin peptides were manually searched for parent ions with an added mass of 72 for aged soman, or an added mass of 108 for aged CPO, as well as for parent ions with an added mass of 162 for nonaged soman and 136 for nonaged CPO. No masses for aged OP adducts were found, though the data sets did contain masses for nonaged adducts. In conclusion, no evidence for aging of the OP-tyrosine adducts of transferrin was found.

Motif for OP Binding to Tyrosine

Comparison of the sequences of the transferrin peptides that bind OP shows no definite consensus sequence around the tyrosine to which the OP binds. What the peptides do have in common is the presence of a positively charged Arginine, Lysine, or Histidine within one to five amino acids from the labeled tyrosine, or within a certain distance in the tertiary structure. Figure 6 shows a distance of 3.37 Å between Tyr 238 and Lys 239, and a distance of 4.06 Å between Tyr 574 and His 535 in the tertiary structure of apo-transferrin. Nearby positively charged residues probably interact with the phenolic hydroxyl group of tyrosine to lower the pKa. Tyrosines with a lower pKa value would be better nucleophiles and thus be better able to attack OP.

Peptide ArgTyrThrArg Covalently Binds OP

The hypothesis was tested that a positively charged amino acid located near a tyrosine could activate tyrosine, enabling it to covalently bind OP. Incubation of peptide ArgTyrThrArg with 0.1, 0.2, or 1mM CPO, dichlorvos, or DFP in pH 8.3 buffer at 37°C for 16 h resulted in covalent labeling of tyrosine (Fig. 7). Thirty-seven percent of a 170μM solution of ArgTyrThrArg was labeled by 1mM DFP, 24% by 1mM

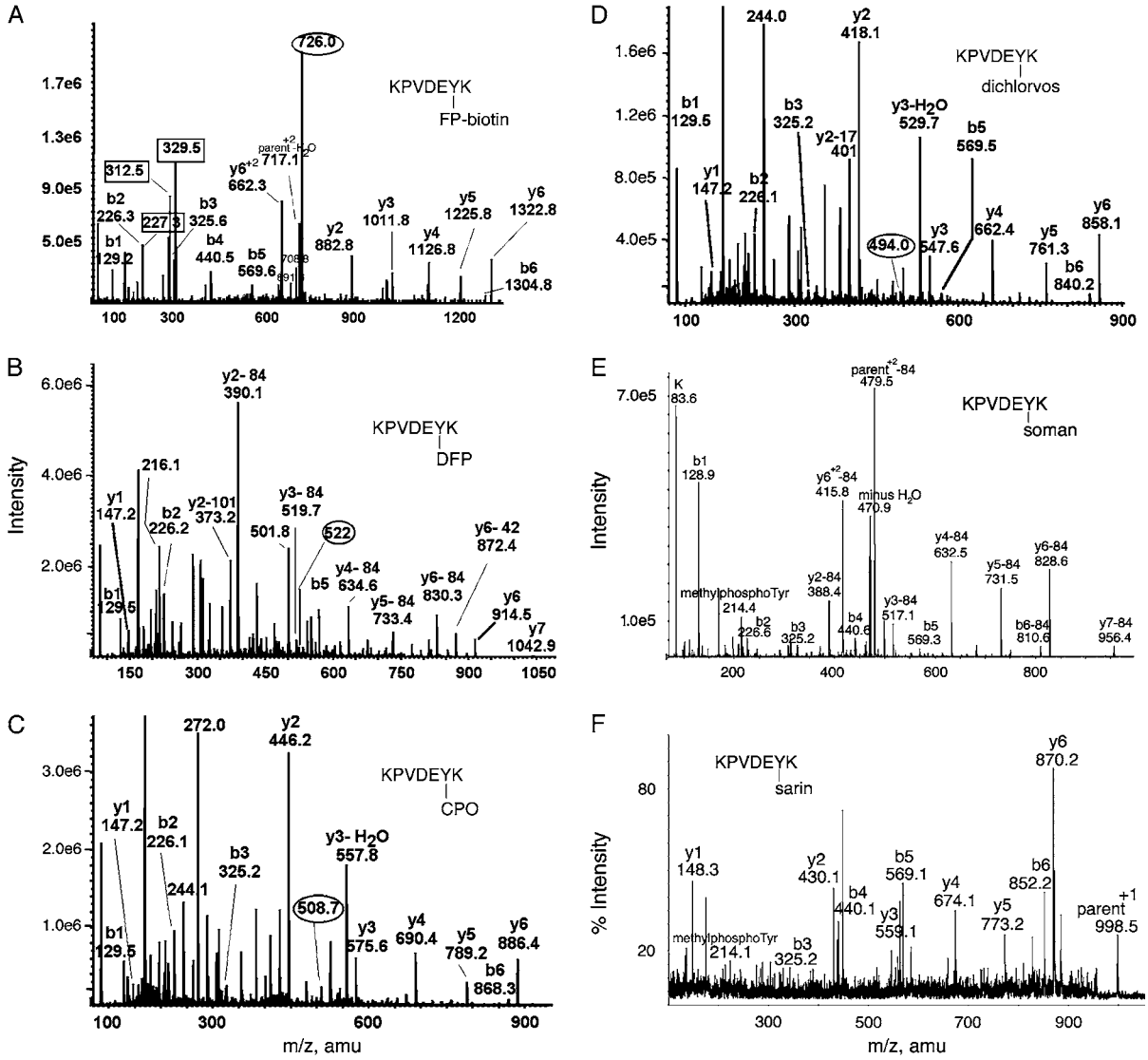

FIG. 4. MS/MS spectra of OP labeled Tyr 238 in peptide KPVDEYK of human transferrin. Mass spectra in (*A-D*) were acquired on the QTRAP 4000 mass spectrometer by infusion, in panel E on the QTRAP 2000 by LC/MS/MS, and in panel F on the MALDI-TOF-TOF mass spectrometer. The b and y ion masses in all panels are consistent with OP covalently bound to Tyr 238. (A) The doubly charged parent ion of the FP-biotin–labeled peptide is at 726.0 *m/z*. Masses enclosed in boxes at 227.3, 312.5, and 329.5 amu are fragments of FP-biotin. The immonium ion of FP-biotinylated tyrosine is at 708.8 amu. Its partner ion at 691.6 amu has lost 17 amu. (B) The doubly charged parent ion of the DFP-labeled peptide is at 522.0 *m/z*. Loss of one or both isopropyl groups during collision-induced dissociation yields y-ions minus 42 or minus 84 amu, confirming the presence of diisopropylphosphate. Loss of both isopropyl groups is the most common observation (Grigoryan *et al*., 2008; Li *et al*., 2007). The y-ion series (y1 and y2–84 through y6-84) indicates that tyrosine is labeled. The delta mass (242.9 amu) between y1 (147.2 amu) and y2–84 (390.1 amu) is consistent with the appearance of tyrosine phosphate at fragment y2 (163 amu for tyrosine and 80 amu for phosphate). The mass at 373.2 amu is the y2 ion minus 101 amu, representing loss of two isopropyl groups as well as ammonia. The mass at 501.8 *m/z* is the doubly charged parent ion minus one isopropyl group. The mass at 216.1 amu is consistent with the phosphotyrosine immonium ion. (C) The doubly charged parent ion of the chlorpyrifos oxon-labeled peptide is at 508.7 *m/z*. The y-ion series (y1–y6) shows the presence of all residues. The mass difference (299.0 amu) between y1 (147.2 amu) and y2 (446.2 amu) clearly shows the diethoxyphosphate on tyrosine in fragment y2 (163 amu for tyrosine and 136 amu for diethoxyphosphate). The mass at 244.1 amu is consistent with the monoethoxyphosphotyrosine immonium ion. The mass at 272.0 amu is consistent with the diethoxyphosphotyrosine immonium ion. (D) The doubly charged parent ion of the dichlorvos-labeled peptide is at 494.0 *m/z*. The y-ion series (y1–y6) shows the presence of all residues. The mass difference (270.9 amu) between y1 (147.2 amu) and y2 (418.1 amu) clearly shows the dimethoxyphosphate on tyrosine in fragment y2 (163 amu for tyrosine and 108 amu for dimethoxyphosphate). The mass at 244.0 amu is consistent with dimethoxyphosphotyrosine immonium ion. (E) The parent ion of the soman-labeled peptide has a mass to charge ratio of 520.7, but this mass does not appear in the scan. The prominent peak at 479.5 is the doubly charged parent ion that has lost the pinacolyl group from soman. The y2–y7 ions have all lost 84 amu due to release of pinacolyl from soman. The peak at 214.4 is the methylphosphotyrosine immonium ion. (F) The parent ion of the sarin-labeled peptide is the singly charged ion at 998.5 amu. The masses of the y-ion series, and the 214.1 mass for methylphosphotyrosine immonium, support labeling on tyrosine.

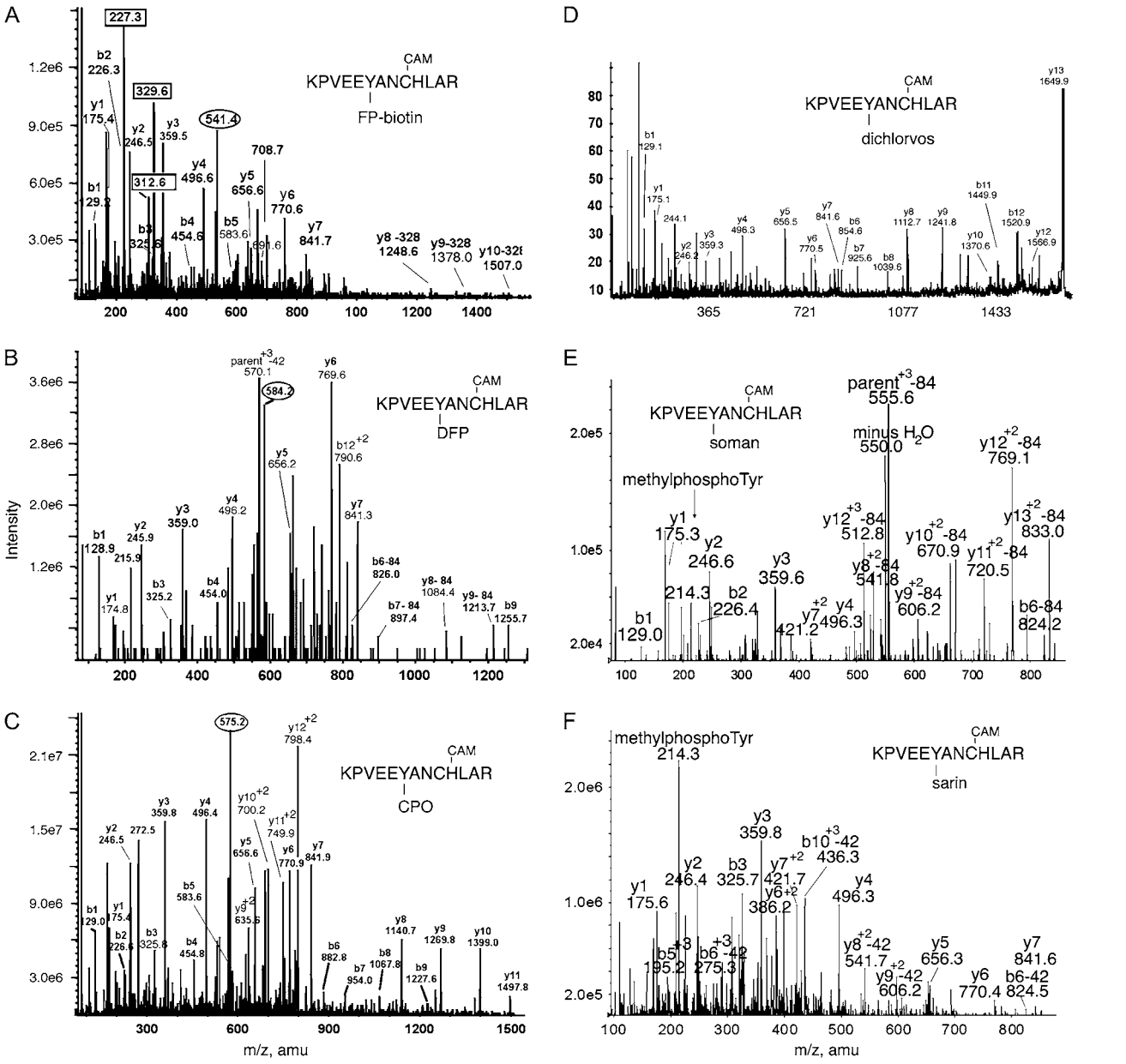

FIG. 5. MS/MS spectra of OP labeled Tyr 574 in peptide KPVEEYANCHLAR of human transferrin. Mass spectra in (A), (B), and (C) were acquired on the QTRAP 4000 mass spectrometer by infusion, in (D) on the MALDI-TOF-TOF, in panels E and F by LC/MS/MS on the QTRAP 2000 mass spectrometer. The b and y ion masses in all panels are consistent with OP covalently bound to Tyr 574. Cysteine has been CAM, which adds a mass of 57 amu. (A) The quadruply charged parent ion of the FP-biotin–labeled peptide is at 541.4 *m/z*. Masses enclosed in boxes at 227.3, 312.6, and 329.6 amu, are fragments of FP-biotin. The immonium ion of FP-biotinylated tyrosine is at 708.7 amu. Its partner ion at 691.6 amu has lost 17 amu. Three y-ions have lost 328 amu from FP-biotin. (B) The triply charged parent ion of the DFP-labeled peptide is at 584.2 *m/z*. Loss of one or both isopropyl groups yields ions minus 42 or minus 84 amu. The y-ion series (y1–y7) supports the identification of the peptide and indicates that the OP label is not in that portion of the peptide. The delta mass (243.2 amu) between y7 (841.3 amu) and y8-84 (1084.4 amu) fits with the appearance of tyrosine-phosphate in fragment y8 (163 amu for tyrosine and 80 amu for phosphate). The presence of tyrosine-phosphate is expected for tyrosine-diisopropylphosphate that has lost both isopropyl groups. Masses at 1213.7 (y9-84), 826.0 (b6-84) and 897.4 amu (b7-84) support this assignment. The mass at 215.9 amu is consistent with the phosphotyrosine immonium ion. (C) The triply charged parent ion of the chlorpyrifos oxon-labeled peptide is at 575.2 *m/z*. The y-ion series (y1–y11) shows a delta mass (299.4 amu) between y7 (841.3 amu) and y8 (1140.7 amu) that is consistent with the appearance of tyrosine-diethoxyphosphate at fragment y8 (163 amu for tyrosine and 136 amu for diethoxyphosphate). This is supported by the b-ion series (b1–b9) which shows the appearance of the same tyrosine as tyrosine-diethoxyphosphate at fragment b6. The mass at 272.5 amu is consistent with the diethoxyphosphotyrosine immonium ion. (D) The singly charged parent ion of the dichlorvos-labeled peptide is at 1694.9 *m/z*. This is a MALDI-TOF-TOF spectrum, where parent ions are typically singly charged. The y-ion series (y1–y10) shows a delta mass (271.1 amu) between y7 (841.6 amu) and y8 (1112.7 amu) which is consistent with the appearance of tyrosine-dimethoxyphosphate at fragment y8 (163 amu for tyrosine and 108 amu for dimethoxyphosphate). The mass at 244.1 amu is consistent with dimethoxyphosphotyrosine immonium ion. (E) The triply charged parent ion of the soman-labeled peptide has a mass of 583.9, but this mass does not appear in the

TABLE 1
Human and Mouse Transferrin (Swiss Prot accession# P02787 and Q921I1) Labeled with OP Agents

OP	Human-labeled peptide	Mouse-labeled peptide	OP-Tyr
FP-biotin	KPVDEY*K	KPVDQY*EDCYLAR	Y238
FP-biotin		LYLGHNY*VTAIR	Y319
FP-biotin		GY*YAVAVVK	Y429
FP-biotin		FDEFFSQGCAPGY*EK	Y491
FP-biotin		EEYNGY*TGAFR	Y518
FP-biotin	KPVEEY*ANCHLAR		Y574
DFP	KPVDEY*K	KPVDQY*EDCYLAR	Y238
DFP		GY*YAVAVVK	Y429
DFP	KPVEEY*ANCHLAR		Y574
CPO	KPVDEY*K	KPVDQY*EDCYLAR	Y238
CPO		LYLGHNY*VTAIR	Y319
CPO		GY*YAVAVVK	Y429
CPO		FDEFFSQGCAPGY*EK	Y491
CPO		EEYNGY*TGAFR	Y518
CPO	KPVEEY*ANCHLAR		Y574
Dichlorvos	KPVDEY*K		Y238
Dichlorvos	KPVEEY*ANCHLAR		Y574
Sarin	KPVDEY*K	KPVDQY*EDCYLAR	Y238
Sarin		GY*YAVAVVK	Y429
Sarin		GYY*AVAVVK	Y430
Sarin	KPVEEY*ANCHLAR		Y574
Soman	KPVDEY*K	KPVDQY*EDCYLAR	Y238
Soman		GYY*AVAVVK	Y430
Soman	KPVEEY*ANCHLAR		Y574

Note. Single letter codes for amino acids are A, ala; C, cys; D, asp; E, glu; F, phe; G, gly; H, his; I, ile, K, lys; L, leu; M, met; N, asn; P, pro; Q, gln; R, arg; S, ser; T, thr; V, val; W, trp; Y, tyr. The labeled tyrosine is designated by an asterisk Y*. The table shows the amino acid sequences of the tryptic peptides containing OP-labeled tyrosine. The location of the labeled tyrosine in the transferrin protein is given in the last column. Tyrosine 238 of transferrin was labeled in both species. Tyr 319, 429, 430, 491, and 518 was labeled only in mouse transferrin. Tyr 574 was labeled only in human transferrin. Transferrin 1 mg/ml in pH 8.5 buffer was treated in vitro with 0.5mM OP at 37°C for 16 h.

CPO, and 21% by 1mM dichlorvos. MS/MS spectra showed that the OP-labeled residue in peptide ArgTyrThrArg was tyrosine. Peptide SerTyrSerMet was also labeled by 1mM DFP, CPO, and dichlorvos, but no labeling was detected with 0.1 and 0.2mM OP. MS/MS spectra showed that the OP-labeled residue in SerTyrSerMet was tyrosine. Peptide SerTyrSerMet ionized poorly in the MALDI-TOF, giving a weak signal with large standard deviation. It was concluded that tyrosines in general could be labeled by OP, but that the most reactive tyrosines were located near a positively charged arginine or lysine.

The higher reactivity of DFP compared with other OP has been explained by formation of a hydrogen-bonded intermediate between fluoride and the hydroxyl group of tyrosine (Ashbolt and Rydon, 1957).

Function of Transferrin is not Disrupted by OP Binding

It was unknown whether covalent binding of an OP to transferrin affected the ability of transferrin to bind ferric ions. Tyrosines 95, 188, 426, and 517 are involved in the binding of ferric ions in human transferrin (Sargent *et al*., 2005).

Human apo-transferrin, labeled with FP-biotin on Tyr 238 and Tyr 574, as well as control apo-transferrin were titrated with ferric nitriloacetate to determine binding stoichiometry. Figure 8 shows that both transferrin preparations bound approximately 2 mol of ferric ion per mole of transferrin, a result in agreement with the known number of ferric ion binding sites in transferrin (Bates and Schlabach, 1973). It was concluded that modification of human transferrin by FP-biotin did not interfere with binding of ferric ion.

DISCUSSION

Serine Hydrolases are not the only OP-Binding Proteins in Human Plasma

Our expectation when we started this project was that OP-labeled proteins would all be serine esterases and serine proteases. We expected butyrylcholinesterase to be the dominant OP-binding protein in human plasma (Fidder *et al*., 2002; Van Der Schans *et al*., 2004). Our results show that this expectation was not met. OP bind not only to serine esterases and serine proteases, but also to proteins that have an activated tyrosine. Using mass spectrometry, we have conclusively demonstrated OP binding to 18 tyrosines in five proteins: human albumin (Ding *et al*., 2008; Li *et al*., 2007), alpha- and beta-tubulin (Grigoryan *et al*., 2008), human transferrin and mouse transferrin. In addition we have shown that human alpha-1-antitrypsin, human complement C3, human alpha-2-macroglobulin covalently bind FP-biotin, though the site of attachment is unknown. Tyrosine in small synthetic peptides was also the site of attachment of OP.

New OP-Binding Motif

The OP-binding motif in the serine hydrolase family is GlyXSerXGly where OP is covalently bound to serine. Though OP binding to Tyr 411 of human albumin has been recognized (Li *et al*. 2007, 2008; Williams *et al*., 2007), OP binding to

spectrum. The mass at 555.5 is the triply charged parent ion that has lost 84 amu from the pinacolyl group of soman. The doubly charged y8–y13 ions have all lost 84 amu from soman; the y8–y13 masses support labeling on tyrosine. The methylphosphotyrosine immonium ion is present at 214.3 amu. (F) The quadruply charged parent ion for sarin-labeled peptide has a mass of 427.7, but this mass does not appear in the spectrum. The most prominent peak is the methylphosphotyrosine immonium ion at 214.3 amu, supporting labeling of tyrosine by sarin. The doubly charged y8 and y9 ions, and the triply charged b5 and b6 ions also support labeling on tyrosine. These ions have lost 42 amu due to loss of the isopropyl group from sarin.

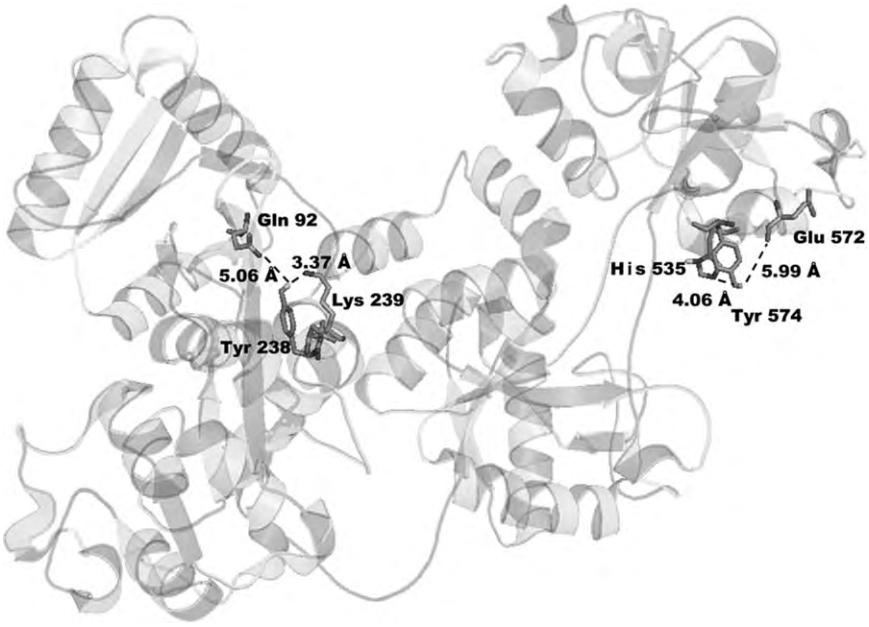

FIG. 6. OP-labeled Tyr 238 and Tyr 574 in the crystal structure of human apo-transferrin (PDB code 2hav). The phenolic hydroxyl of Tyr 238 is 3.37 Å from the amine of Lys 239 and 5.06 Å from Gln 92. The phenolic hydroxyl of Tyr 574 is 4.06 Å from the imidazole nitrogen of His 535 and 5.99 Å from oxygen in the carbonyl of Glu 572 (Wally *et al.*, 2006).

tyrosine as a general phenomenon has not been appreciated. Our results show that OP binding to tyrosine is not limited to Tyr 411 of albumin, but is found on many tyrosines in many proteins. Even the small peptide ArgTyrThrArg made a covalent bond with OP. Our analysis of OP-labeled tyrosine peptides reveals no consensus amino acid sequence around the labeled tyrosine. The chief requirement for OP binding to tyrosine appears to be a nearby positively charged arginine or lysine. The positively charged groups stabilize the phenolate anion of tyrosine, thus lowering the pKa of tyrosine. The unusually low pKa enables the negatively charged phenolic anion to react with OP at physiological pH values.

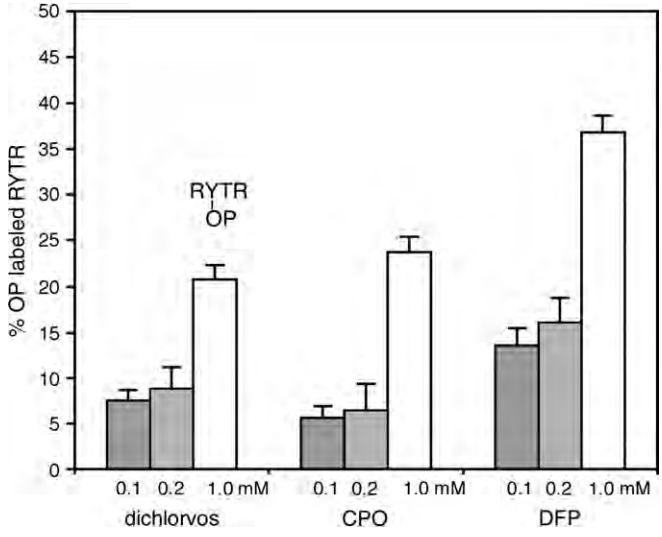

FIG. 7. Labeling of peptide ArgTyrThrArg by dichlorvos, chlorpyrifos oxon, and DFP. The reaction of 170μM peptide with 0.1, 0.2, or 1mM OP was in ammonium bicarbonate buffer pH 8.3, at 37°C for 16 h. MALDI-TOF cluster areas were used to calculate % labeling. MS/MS spectra identified the labeled residue as tyrosine.

It is anticipated that many more proteins than those we have already identified can be modified by OP. Proteins with no active site serine have been implicated in OP-induced neurodevelopmental, behavioral, and immunological effects. These proteins include neurotransmitter receptors (Aldridge *et al.*, 2003; Bomser and Casida, 2001; Katz *et al.*, 1997; Lein and Fryer, 2005; Pope, 1999; Quistad *et al.*, 2002; Smulders *et al.*, 2004), proteins in the adenylyl cyclase signaling cascade (Song *et al.*, 1997), cyclic AMP response element binding protein (Schuh *et al.*, 2002), immune function proteins (Kassa *et al.*, 2003), and kinesin in the axonal transport system (Gearhart *et al.*, 2007; Prendergast *et al.*, 2007; Terry *et al.*, 2007). Whether OP bind to tyrosine in these proteins is unknown.

Biomarkers of OP Exposure

Our in vitro conditions used high concentrations of OP that would not be found during *in vivo* poisoning. It is unknown whether live animals treated with a nonlethal dose of OP would have OP-labeled transferrin. However, it is known that mice treated with a nontoxic dose of FP-biotin (1 and 5 mg/kg ip) (Peeples *et al.*, 2005) and guinea pigs treated with sarin, soman, cyclosarin, and tabun have OP-labeled albumin in their blood (Williams *et al.*, 2007). The guinea pigs received 0.5–5 LD_{50} doses of nerve agent, but were alive up to 7 days later

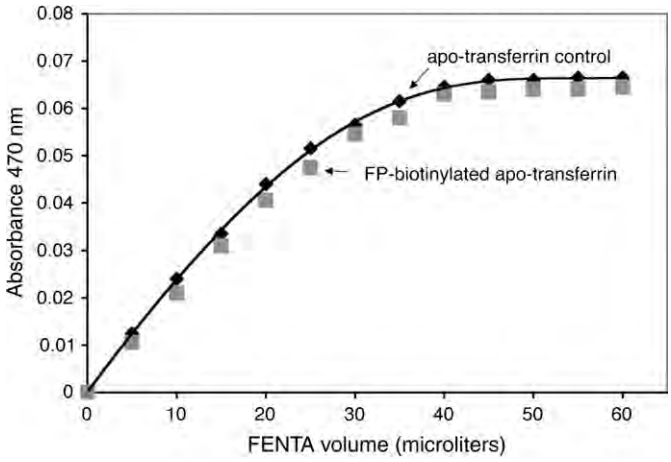

FIG. 8. FP-biotinylation of Tyr 238 and Tyr 574 in human transferrin does not interfere with ferric ion binding. One milliliter of 12.1μM apo-transferrin control (♦) titrated with FENTA had an endpoint of 27.3μM FENTA, which calculates to 2.26 mol of ferric ion bound per mole of transferrin. One milliliter of 14.67 μM FP-biotinylated apo-transferrin (■) had an endpoint of 31.3μM FENTA, which calculates to 2.11 mol of ferric ion bound per mole of transferrin. Assays for each type of transferrin were performed in duplicate.

because they had been protected with pyridostigmine, oximes, atropine, and midazolam. Tyrosine-modified proteins could serve as biomarkers for OP exposure.

No Aging

The serine hydrolases, acetylcholinesterase, butyrylcholinesterase, and acylpeptide hydrolase have been shown to serve as biomarkers of OP exposure in mice and rats (Quistad *et al*., 2005; Richards *et al*., 2000). OP-labeled serine hydrolases lose part of the OP in a process called aging, making it impossible to distinguish between exposure to sarin and soman. In contrast, OP-labeled tyrosines do not undergo aging, so that labeling by sarin is clearly distinguished from labeling by soman.

Significance

Our findings may have application to diagnosis and treatment of OP exposure. Proteins that have no active site serine may serve as biomarkers of exposure. In the future it may be possible to develop antibodies to the new OP-labeled biomarkers to use for screening OP exposure. Synthetic peptides containing arginine, lysine, and tyrosine may find application as scavengers to clear the body of OP. The recognition of a new OP-binding motif to tyrosine suggests new directions to search for mechanisms of long-term effects of OP exposure.

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Carbofuran poisoning detected by mass spectrometry of butyrylcholinesterase adduct in human serum

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ABSTRACT: Carbofuran is a pesticide whose acute toxicity is due to inhibition of acetylcholinesterase. Butyrylcholinesterase (BChE) in plasma is inhibited by carbofuran and serves as a biomarker of poisoning by carbofuran. The goal was to develop a method to positively identify poisoning by carbofuran. Sera from an attempted murder and an attempted suicide were analyzed for the presence of carbofuran adducts on BChE. The BChE from 1 ml of serum was rapidly purified on a 0.2 ml procainamide–Sepharose column. Speed was essential because the carbofuran–BChE adduct decarbamylates with a half-life of about 2 h. The partially purified BChE was boiled to denature the protein, thus stopping decarbamylation and making the protein vulnerable to digestion with trypsin. The labeled peptide was partially purified by HPLC before analysis by LC/MS/MS in the multiple reaction monitoring mode on the QTRAP 2000 mass spectrometer. Carbofuran was found to be covalently bound to Ser 198 of human BChE in serum samples from two poisoning cases. Multiple reaction monitoring triggered MS/MS spectra positively identified the carbofuran–BChE adduct. In conclusion a mass spectrometry method to identify carbofuran poisoning in humans has been developed. The method uses 1 ml of serum and detects low-level exposure associated with as little as 20% inhibition of plasma butyrylcholinesterase. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: carbofuran; butyrylcholinesterase; multiple reaction monitoring; pesticide; carbamate; human plasma

Introduction

Carbofuran (Fig. 1) is a carbamate pesticide used to control beetles, nematodes and rootworm in fields of alfalfa, rice, grapes and sugar beet (Gupta, 1994). The acute toxicity of carbofuran for both insects and mammals is due to inhibition of acetylcholinesterase. In people, activity assays of red blood cell acetylcholinesterase and serum butyrylcholinesterase (BChE) can verify that poisoning has occurred but cannot identify the type of poison. Gas-chromatography followed by mass spectrometry has been used to detect carbofuran metabolites in plasma and urine (Barr *et al*., 2002; Petropoulou *et al*., 2006a, 2006b). However, intact carbofuran has been difficult to find because it is rapidly degraded in plasma and liver. The presence of carbofuran metabolites in plasma and urine could mean that a person ingested or inhaled the metabolites rather than the parent compound. An alternative strategy to obtain proof of exposure takes advantage of the fact that carbofuran makes a covalent bond with the active site serine of butyrylcholinesterase (Fig. 2). Metabolites do not make this covalent bond. Therefore, identification of the methylcarbamate–butyrylcholinesterase adduct in a person's serum provides conclusive proof that the individual has been exposed to a carbamate.

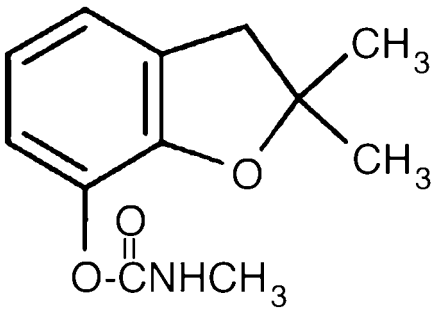

Figure 1. Structure of carbofuran.

This is the first report to describe a mass spectrometry method for detection of the methylcarbamate–BChE adduct. The method extends previous work on detection of organophosphorus–BChE adducts in human plasma (Fidder *et al*., 2002).

Carbamates are used to slow the progression of Alzheimer's disease (Weinstock and Groner, 2008). Toxicity from these drugs could be monitored by the mass spectrometry method described in this report.

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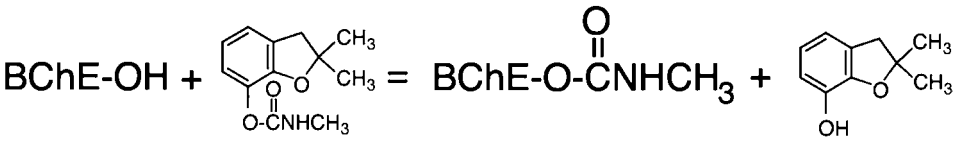

Figure 2. Covalent binding of carbofuran to BChE. The active site serine 198 of human BChE makes a covalent bond with the carbamate group of carbofuran, releasing the hydroxybenzofuran ring structure. The mass added to BChE by the carbamate is 57.05 amu.

Methods

Materials

Human BChE was purified from 70 l of outdated human plasma by ion exchange chromatography at pH 4.0, followed by procainamide affinity chromatography, and ion exchange chromatography at pH 7.4 (Lockridge *et al*., 2005). The 574 amino acid sequence of the secreted human BChE protein plus the 28 amino acid signal peptide sequence for a total of 602 amino acids are in file accession no. gi: 116353. Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol *N*-methyl carbamate) was from Aldrich (no. 426008). Carbofuran was dissolved in dimethylsulfoxide just before use. Sequencing grade modified trypsin (Promega V5113) at a concentration of 20 μg in 50 μl of 50 mM acetic acid was stored at −80 °C.

Pure Human BChE Labeled with Carbofuran for Mass Spectrometry

An aliquot of 87 μl of 1 mg/ml BChE (1 nmole) in 10 mM ammonium bicarbonate was treated with 5 μl of freshly prepared 0.4 mM carbofuran (2 nmol) for 30 min at room temperature. BChE activity fell from 720 to 122 u/ml, indicating 83% inhibition. A BChE control sample was prepared in the same way except that exposure to carbofuran was omitted. Both the carbofuran-treated and the control BChE samples were boiled for 10 min in a water bath to denature the protein in preparation for digestion with 5 μl of 0.4 μg/μl trypsin. Digestion was at 37 °C for 22 h. The active site peptide was purified using a Phenomenex Prodigy, 5 μm, ODS(2) C_{18} column (100 × 4.60 mm) on a Waters HPLC system. Peptides were eluted with a 60 min gradient starting at 0.1% trifluoroacetic acid, and ending at 60% acetonitrile–40% 0.1% trifluoroacetic acid, at a flow rate of 1 ml/min. A 0.5 μl aliquot from each 1 ml fraction was analyzed by MALDI-TOF mass spectrometry to identify samples containing the active site peptide. HPLC fractions were dried in a vacuum centrifuge and redissolved in 100 μl of 50% acetonitrile, 0.1% formic acid in preparation for infusion into the QTRAP 4000 tandem quadrupole mass spectrometer, or in 100 μl of 5% acetonitrile, 0.1% formic acid for LC/MS/MS on the QTRAP 2000 tandem quadrupole mass spectrometer.

BChE Activity

BChE activity was assayed with 1 mM butyrylthiocholine iodide in 0.1 M potassium phosphate pH 7.0 in the presence of 0.5 mM dithiobisnitrobenzoic acid, at 25 °C by measuring the increase in absorbance at 412 nm. The slope per min was converted to μmoles per min using the extinction coefficient $E = 13\,600\ M^{-1}\,cm^{-1}$ (Ellman *et al*., 1961).

Serum

Serum samples (no anticoagulant) from an attempted murder and an attempted suicide were obtained from Professor Ivan Ricordel, Paris Police. Samples were shipped to Nebraska on dry ice and stored at −80 °C.

The attempted murder victim was a 63-year-old man who had been repeatedly poisoned. He was admitted to the Intensive Care Unit for respiratory failure requiring mechanical ventilation. Clinical examination revealed myosis, hypersialorrhea and some paralysis. Plasma cholinesterase activity measured 6 and 17 h after admission was 2.5 and 4.8 U/ml (normal: 7–9 U/ml). Clinical symptoms improved rapidly, allowing extubation by 12 h. The rapid recovery was consistent with poisoning by a carbamate rather than an organophosphorus agent. Hair analysis revealed the presence of carbofuran and its main metabolite, 3-hydroxycarbofuran (Dulaurent *et al*., 2008).

The suicidal female patient had ingested 20 mg of carbofuran (Curater 5%) and alcohol. Diagnosis of the nature of the toxicant was based on interview of the patient and the pesticide bottle brought to the hospital. The patient had abdominal pain, but no other cholinergic symptoms of toxicity. Her alcohol level was 1.37 g/l. She was treated with atropine to block muscarinic receptors, and with rivotril for anxiety. The interval between exposure and blood sampling was 7 h. After centrifugation to remove coagulated blood, the serum was frozen at −30 °C until it was shipped on dry ice.

Purification of BChE from 1 ml Serum

Human serum contains about 0.05 nmol of BChE in 1 ml. This amount was expected to be detectable in the mass spectrometer, based on studies with highly purified BChE. BChE from 1 ml serum was purified by passage over 0.2 ml of procainamide affinity gel packed in a 1.5 ml microfuge spin column. The affinity gel had been custom synthesized by Dr Yacov Ashani (Grunwald *et al*., 1997) and had a specific activity of 34 μmol procainamide bound per ml Sepharose. The gel was equilibrated with 2 ml of 20 mM potassium phosphate pH 7.0 buffer. One milliliter of serum was loaded on the column by gravity flow at a rate of 1 ml in 10 min. Centrifugation was not used for this step because binding was more complete when loading was slow. The column was washed four times with 1 ml of 0.2 M NaCl in 20 mM potassium phosphate pH 7.0 buffer. The wash time was reduced to less than 0.5 min per ml by briefly centrifuging the spin column. The column was eluted three times with 0.5 ml of 1 M sodium chloride in 20 mM potassium phosphate pH 7.0 buffer to remove the BChE. The total time elapsed from thawing the plasma to elution was 15 min. Speed was critical because the carbamate is easily released from intact BChE. A 10 μl aliquot of each fraction was saved for nondenaturing gel electrophoresis.

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Trypsin Digestion and HPLC Purification of the Carbofuran–BChE Peptide

The 0.5 ml BChE fraction in 1 M NaCl containing the majority of the BChE was immediately boiled for 10 min to denature the protein, and thus prevent decarbamylation of the active site serine. A second reason for boiling the BChE was to unfold the protein to allow trypsin access to cleavage sites that otherwise are blocked by the sugar-coated surface of the BChE molecule. There was no need to reduce and alkylate disulfide bonds. The BChE was digested with 20 μl of 0.4 μg/μl trypsin for 18 h at 37 °C. The active site peptide was partially purified on a Waters HPLC as described above. The sample that eluted between 34 and 35% acetonitrile was analyzed in the QTRAP 4000 and QTRAP 2000 mass spectrometers.

Nondenaturing Gel Electrophoresis

A 4–30% polyacrylamide gradient gel was prepared in a Hoefer gel apparatus (Hoefer became Pharmacia then Amersham and is now GE Healthcare). Electrophoresis was for 5000 V h (250 V, 20 h) at 4 °C. Bromophenol Blue eluted off the gel long before electrophoresis was stopped. The gel was stained for BChE activity with butyrylthiocholine by the method of Karnovsky and Roots, and counterstained with Coomassie blue (Karnovsky and Roots, 1964).

Infusion into the QTRAP 4000 Mass Spectrometer

HPLC fractions containing the partially purified carbofuran-labeled BChE peptide were infused into the QTRAP 4000 hybrid, quadrupole, linear ion-trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) at a flow rate of 0.3 μl/min through an 8 μm emitter (no. FS360-50-8-D, New Objective) via a 25 μl Hamilton syringe mounted on a Harvard syringe pump. An ion spray potential of 1900 V was maintained between the emitter and the mass spectrometer. Selected masses were fragmented by collision induced dissociation in a collision cell pressurized to 40 μTorr with pure nitrogen, using a collision energy of 40 eV. The mass spectrometer was calibrated on selected fragments from the MS/MS spectrum of Glu Fibrinopeptide B. 500 MS/MS scans were summed to produce the final spectrum. The sequence of the peptide was determined by manual inspection of the fragmentation spectra.

Multiple Reaction Monitoring on the QTRAP 2000 Mass Spectrometer

Ten microliters of HPLC-purified, tryptic peptides were injected onto a nanocolumn (no. 218MS3.07515 Vydac C_{18} polymeric rev-phase, 75 μm i.d. × 150 mm long; P.J. Cobert Assoc, St Louis, MO, USA) for a second phase of HPLC separation. Peptides were separated with a 90 min linear gradient from 0 to 60% acetonitrile at a flow rate of 0.3 μl/min and electrosprayed through a fused silica emitter (360 μm o.d., 75 μm i.d., 15 μm taper, New Objective) directly into the QTRAP 2000, a hybrid quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA). An ion-spray voltage of 1900 V was maintained between the emitter and the mass spectrometer. The collision cell was pressurized to 40 μTorr with pure nitrogen and collision energies between 20 and 40 eV were used. The collision energy for a given peptide was determined automatically by the Analyst software, based on the mass and charge of the precursor ion. The mass spectrometer was calibrated on selected fragments from the MS/MS spectrum of Glu-Fibrinopeptide B. The MS/MS data were collected and processed using Analyst 1.4.1 software (Applied Biosystems).

The QTRAP 2000 was operated in MRM mode. MRM transition ions were selected based on the fragmentation pattern of the carbofuran-labeled BChE active site peptide determined from the infusion experiments. The Q1 mass was set to 747.6 *m/z* for the quadruply-charged parent ion SVTLFGES*AGAASVSLHLLSPGSHSLFTR, where the labeled Ser198 is indicated by an asterisk. The Q3 masses were set to 1001.5 amu for the singly-charged y9 ion, and to 1201.6 amu for the singly-charged y11 ion. The BChE peptide AILQSGSFNAPWAVTSLYEAR co-purified with the active site peptide. It was included in the MRM search as a positive control. The Q1 mass for that peptide was set to 761.5 *m/z* for the triply-charged parent ion. The Q3 masses were set to 839.4 amu for the singly-charged y7 ion, and to 1009.5 amu for the singly-charged y9 ion. The reference peptide served as assurance that the MRM method was working.

MALDI-TOF-TOF 4800 Mass Spectrometer (Applied Biosystems, Foster City, CA, USA)

Essentially salt-free 0.5 μl samples from the offline HPLC fractions were spotted on a MALDI target plate, air dried and overlaid with 0.5 μl of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid. MS spectra were acquired with laser power at 3000 V in positive reflector mode. The mass spectrometer was calibrated against des-Arg-Bradykinin, Angiotensin 1, Glu Fibrinopeptide B and neurotensin (Cal Mix 1, Applied Biosystems). Each spectrum was the average of 500 laser shots.

Decarbamylation Rate of Carbofuran-labeled BChE

Highly purified human BChE with an activity of 540 u/ml (0.75 mg/ml) was dissolved in phosphate buffered saline pH 7.4. A 560 μl aliquot (4.94 nmol) was treated with 1 μl of 10 mM carbofuran (10 nmol) for 1 h at 25 °C, at which time 88% of the BChE was inhibited. A 5 μl aliquot was diluted into 500 μl of phosphate buffered saline to make the solution used for measuring the rate of decarbamylation. Seven replicate dilutions were prepared in seven tubes. At various times after dilution, 20 μl was removed for assay of BChE activity.

Results

Pure BChE Labeled with Carbofuran

HPLC fractions were analyzed by MALDI-TOF to identify the elution positions of the 2985.5 amu carbofuran–BChE peptide and the 2928.5 amu unlabeled active site peptide. The methylcarbamyl-labeled as well as the unlabeled active site tryptic peptides started to elute at 34% acetonitrile and continued to elute with lower intensity to 42% acetonitrile. The highest concentration was in the 34–35% acetonitrile fraction.

The 34–35% fraction, containing the partially purified, methylcarbamyl-labeled peptide was infused into the QTRAP 4000 mass spectrometer where collision-induced dissociation of the triply-charged parent ion at 996.4 *m/z* yielded the MS/MS spectrum in Fig. 3. The b- and y-ion masses in Fig. 3 are consistent with the sequence SVTLFGES*AGAASVSLHLLSPGSHSLFTR where S* has an added mass of 57 due to covalent binding of carbamate. Masses for the doubly-charged ions from $y22^{+2}$ to $y27^{+2}$ are

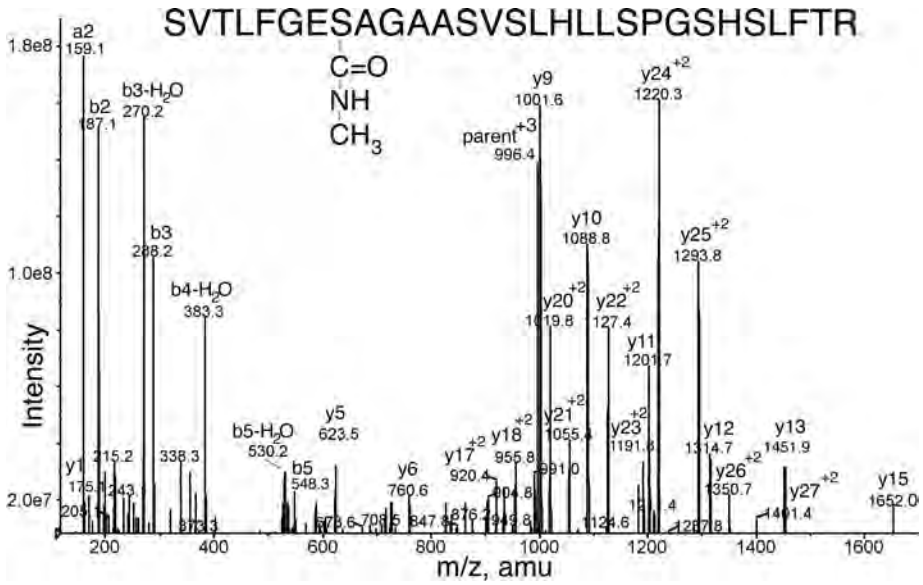

Figure 3. MS/MS spectrum of the carbofuran (methylcarbamyl-)-labeled tryptic peptide from pure human BChE (accession no. gi:116353). The doubly-charged $y22^{+2}$ to $y27^{+2}$ ions carry the carbamate on Ser 198. This increases their mass by 57 amu compared with the unlabeled ions. The spectrum was obtained on the QTRAP 4000 mass spectrometer by infusing purified peptide.

consistent with the presence of carbamate on Ser 198. Carbamate increases each of their masses by 57 amu compared to the unlabeled ions. The $y22^{+2}$ ion (S*AGAASVSLHLLSPGSHSLFTR) is 144 amu heavier than the $y21^{+2}$ ion (AGAASVSLHLLSPGSHSLFTR), a mass that exactly fits Ser (87 amu) plus carbamate (57 amu).

No ions for dehydrated serine were found. Unlike organophosphorus-modified serine (Fidder *et al*., 2002; Tsuge and Seto, 2006; Sun and Lynn, 2007) and pyridostigmine (dimethylcarbamyl)-modified serine (Fidder *et al*., 2002), carbofuran (methylcarbamyl)-modified serine did not undergo beta-elimination during fragmentation when the collision energy was 40 V in the QTRAP mass spectrometer. Beta-elimination would have yielded ions that were missing the carbamate as well as water for a total loss of 75 amu.

Rate of Decarbamylation of Carbofuran (Methylcarbamyl-)-labeled Pure BChE

The half-life for decarbamylation of the carbofuran–BChE adduct was measured by regain of BChE activity as a function of time, during incubation at pH 7.4, 25 °C. Figure 4 shows that the half-life was 142 ± 8 min, which corresponds to a first-order rate constant of $k = 0.004\,88\ \text{min}^{-1}$. A half-life of about 2 h for reactivation of the adduct means that a BChE purification method would have to be rapid if any of the labeled, active site peptide were to be recovered.

Residual BChE Activity in the Serum from Victims of Carbofuran Poisoning

The serum from the attempted murder victim had a BChE activity of 0.5 u/ml, while serum from the attempted suicide had an activity of 2.3 u/ml. The average BChE activity in unexposed serum is about 3.0 u/ml, indicating that BChE from the murder victim was 83% inhibited, while BChE from the suicide attempt was 23% inhibited.

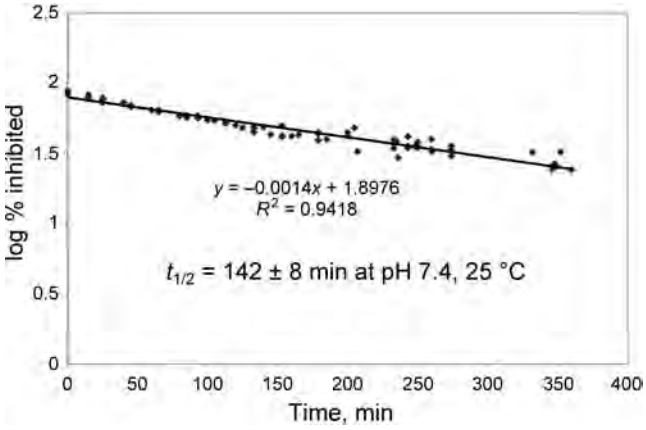

Figure 4. Decarbamylation rate of carbofuran (methylcarbamylated)–BChE. BChE activity was 88% inhibited at time zero, but became less inhibited with time as carbofuran was released from the active site Ser 198. The points are the data, while the line is a linear regression. The equation describes the regression line and R^2 indicates the standard error. The experiment was repeated seven times.

Purification of BChE from 1 ml Serum

Fifty-seven percent of the starting BChE activity was recovered when the procainamide affinity column was eluted with the first 0.5 ml of 1 M sodium chloride. An additional 20% was recovered with the subsequent 1 ml of 1 M sodium chloride. Only 13% of the activity was lost during washing with 0.2 M NaCl (Table 1). The BChE in the first eluate was purified about 60-fold, for the example shown in Table 1. With other samples this purification method has yielded up to 600-fold purified BChE.

The nondenaturing gel stained for BChE activity in Fig. 5(A) shows that the majority of BChE in human serum is a tetramer

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Table 1. Fifteen-minute purification of carbofuran–BChE from 1 ml of serum, on a 0.2 ml procainamide–Sepharose column

Sample	Composition	Volume, ml	Units/ml	Total units	A280	Units/mg
Serum	Serum	1	2.32	2.32	55.7	0.075
Flow-thru	Loading	1	0.071	0.071	39.46	0.003
Wash	0.2 M NaCl	1	0.205	0.205	14.22	0.026
Wash	0.2 M NaCl	1	0.059	0.059	1.492	0.071
Wash	0.2 M NaCl	1	0.024	0.024	0.243	0.178
Wash	0.2 M NaCl	1	0.022	0.022	0.196	0.202
Elute	1 M NaCl	0.5	2.61	1.30	1.060	4.41
Elute	1 M NaCl	0.5	0.98	0.49	0.372	4.74
Elute	1 M NaCl	0.5	0.073	0.037	0.120	1.11

Sodium chloride solutions are in 20 mM potassium phosphate, pH 7, 1 mM EDTA, 0.01% sodium azide.

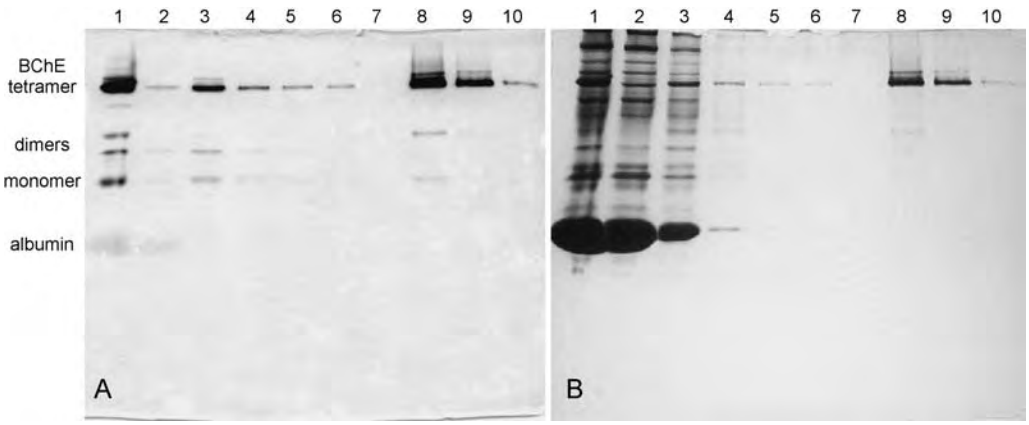

Figure 5. Nondenaturing gel stained for BChE activity (A) and counterstained with Coomassie blue (B). Aliquots of 10 μl of serum and fractions from the affinity column purification of BChE were loaded into each lane of a 4–30% polyacrylamide, gradient gel. Lane 1, serum; lane 2, flow-thru; lane 3, first wash, with 1 ml of 0.2 M sodium chloride; lane 4, second wash; lane 5, third wash; lane 6, fourth wash; lane 7, blank; lane 8, first eluate, with 0.5 ml of 1 M sodium chloride; lane 9, second eluate; lane 10, third eluate.

(lane 1). Two bands of activity appear in the dimer region. The faster migrating dimer is composed of one BChE subunit linked to one albumin molecule through a disulfide bond (Masson, 1989). The BChE–albumin dimer eluted during the wash with 0.2 M NaCl (lane 3) and was not recovered with the partially-purified BChE shown in lane 8. Bromophenol blue binds to human albumin and is visible as a rapidly migrating band in lane 1 of panel A. The same gel was counterstained with Coomassie blue in Fig. 5(B). The most intense Coomassie-staining band is for albumin. The affinity gel did not bind albumin as can be seen by the albumin bands in lanes 2, 3 and 4. No Coomassie blue stained bands are present in lanes 8–10 of Fig. 5(B). The bands that appear in the figure are the brownish red bands of copper ferrocyanide precipitate formed during the activity staining reaction.

Though the BChE appears to be relatively pure in Fig. 5(B), it still is contaminated with many proteins. These contaminating proteins generate peptides during tryptic digestion that interfere with ionization of the BChE active site peptide in the mass spectrometer. Therefore the tryptic digest of the BChE eluate was further purified offline using a Waters HPLC system. The 2985.5 amu peak for the carbofuran-labeled active site peptide of BChE was barely detectable by MALDI-TOF analysis of the HPLC fractions; however from the control experiments we knew that this peptide would elute in the 34–35% acetonitrile fraction. Therefore this fraction was collected and analyzed by tandem, quadrupole mass spectrometry.

Tandem Quadrupole Ion Trap Mass Spectrometry

The purified carbofuran (methylcarbamyl)–BChE peptide that had been isolated from 1 ml of serum was infused into the QTRAP 4000 mass spectrometer. MS/MS spectra for the quadruply-charged parent ion at 747.1 and the triply-charged parent ion at 995.8 had 20–30 singly-, doubly-, and triply-charged ions that fit the carbofuran (methylcarbamyl)–BChE peptide sequence. Both the attempted murder and suicide samples gave spectra consistent with the carbofuran (methylcarbamyl)–BChE peptide. The signal-to-noise ratio ranged from 2 to 10, and peak intensities were about 100-fold lower than for pure BChE.

Multiple Reaction Monitoring

The carbofuran (methylcarbamyl)-labeled peptide from the murder and suicide victims was also characterized by a multiple reaction monitoring (MRM) strategy. The mass spectrometer was instructed to ignore all masses except for the quadruply-charged parent ion at 747.6 *m/z* and two singly-charged daughter ions at

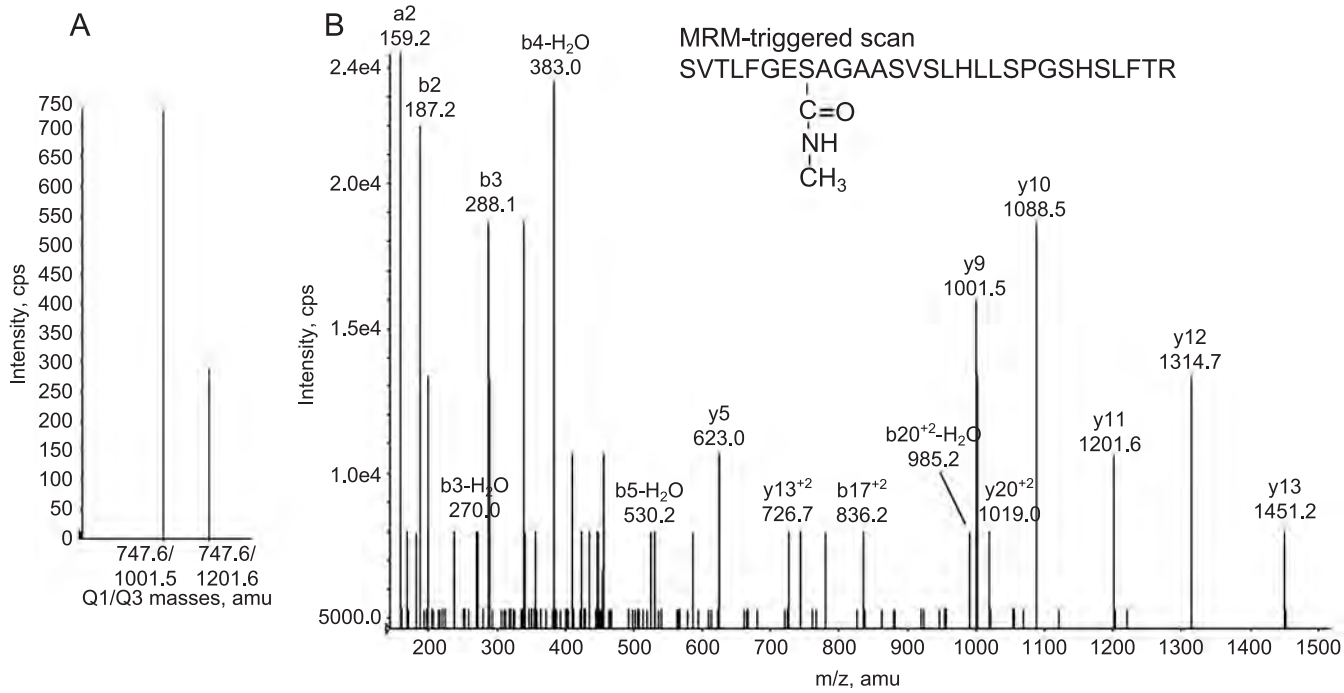

Figure 6. MRM-triggered MS/MS scan of carbofuran (methylcarbamyl)-labeled BChE peptide from 1 ml of serum taken from the attempted murder victim. (A) MRM transitions for the quadruply-charged parent ion at 747.6 *m/z* and singly-charged daughter ions at 1001.5 and 1201.5 amu. (B) The MS/MS spectrum of the 747.6 *m/z* parent ion was triggered by the MRM scan. The MS/MS scan confirms that the parent ion is the carbofuran-labeled active site peptide of human BChE.

1001.5 and 1201.6 amu. These masses are referred to as the Q1 and Q3 transitions [Fig. 6(A)]. When the mass spectrometer identified a peptide with these transitions, it automatically acquired an MS/MS spectrum for that parent ion [Fig. 6(B)].

The MRM-triggered MS/MS scan in Fig. 6(B) has an intensity that is 4 orders of magnitude lower than the MS/MS scan in Fig. 3, and it has fewer ions. Nevertheless, the ions in the MRM-triggered scan have the masses expected for fragments from the carbofuran(methylcarbamyl)-labeled peptide SVTLFGES*AGAASVSLHLL-SPGSHSLFTR. Comparison of the MS/MS spectrum in Fig. 6(B) with that in Fig. 3 revealed 16 ions characteristic of the carbofuran (methylcarbamyl)-labeled BChE active site tryptic peptide. The mass of the parent ion is consistent with the active site peptide of BChE modified with an added mass of 57 amu. The doubly-charged b17 and b20 minus H_2O ions support modification on Ser 198.

Serum samples from both the murder and suicide victims yielded MRM-triggered MS/MS scans that supported the presence of carbofuran-labeled BChE in their sera.

Discussion

Carbamate Adducts on BChE are Unstable

The *N*-methyl carbamate adduct of human BChE has been reported to have a half-life of 11 min at pH 7.4, 37 °C (Wetherell and French, 1991) or 3 h at pH 7.4, 25 °C (Reiner, 1971). In our hands the half-life at pH 7.4, 25 °C was about 2.4 h. The short half-life of the carbofuran adduct meant the BChE had to be purified rapidly to prevent loss of the carbofuran label. This was achieved by purifying BChE on affinity gel packed in a spin column. The short half-life also means there is a need to collect the serum samples in a timely manner from the victims. If several days pass between exposure and serum collection, it is likely that all the carbofuran (methylcarbamyl)–BChE adduct will have been lost.

The *N*-dimethyl carbamate adduct of human BChE (e.g., BChE carbamylated by pyridostigmine or by M7C or by an analog of rivastigmine) is more stable than the *N*-methyl carbamate. The half-life of the *N*-dimethyl carbamalyted BChE is 277 min at pH 7.4, 25 °C, a value that calculates to a rate of decarbamylation of 0.0025 min^{-1} (Lockridge and La Du, 1978). The rate of decarbamylation is 0.0063 min^{-1} at pH 8.0, 37 °C (Weinstock and Groner, 2008).

Decarbamylation is a Catalytic Process

The partially purified BChE was boiled immediately after it was eluted from the affinity column. This step was used because decarbamylation is a catalytic process requiring the native conformation of BChE (Main, 1979), and therefore decarbamylation can be halted by denaturing the enzyme. This concept was proven to be correct by the fact that the carbofuran (methylcarbamyl)-labeled peptide was recovered despite the passage of many days between the time the BChE was boiled and the peptide was analyzed.

Mass Spectrometry to Identify the Poison

The method introduced in this work identifies poisons that add methylcarbamate, a mass of 57 amu, to the BChE active site. Carbofuran is not the only poison that adds a mass of 57 amu. Physostigmine, carbaryl, aldicarb, aldoxycarb, formetanate, methiocarb, methomyl, oxamyl, propoxur and physovenine all

give the same added mass of 57 amu. Analysis of degradation products present in urine can be used to discriminate between these toxins (Barr *et al*., 2002; Petropoulou *et al*., 2006a). However, other carbamates such as pyridostigmine and pirimicarb add dimethylcarbamate to BChE, an added mass of 71 amu. The method described in this report can be used to discriminate between these classes of carbamate toxins. The presence of an adduct on BChE means the person was exposed to the intact carbamate pesticide.

Significance

The presence of metabolites of carbofuran in blood and urine provides evidence of exposure, but this evidence can be interpreted to mean that degradation products rather than the active poison were ingested. Indisputable proof of exposure to active poison is the finding of butyrylcholinesterase adducts.

Acknowledgements

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Covalent binding of the organophosphorus agent FP-biotin to tyrosine in eight proteins that have no active site serine

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ABSTRACT

Organophosphorus (OP) esters are known to bind covalently to the active site serine of enzymes in the serine hydrolase family. It was a surprise to find that proteins with no active site serine are also covalently modified by OP. The binding site in albumin, transferrin, and tubulin was identified as tyrosine. The goal of the present work was to determine whether binding to tyrosine is a general phenomenon. Fourteen proteins were treated with a biotin-tagged organophosphorus agent called FP-biotin. The proteins were digested with trypsin and the labeled peptides enriched by binding to monomeric avidin. Peptides were purified by HPLC and fragmented by collision induced dissociation in a tandem ion trap mass spectrometer. Eight proteins were labeled and six were not. Tyrosine was labeled in human alpha-2-glycoprotein 1 zinc-binding protein (Tyr 138, Tyr 174 and Tyr 181), human kinesin 3C motor domain (Tyr 145), human keratin 1 (Tyr 230), bovine actin (Tyr 55 and Tyr 200), murine ATP synthase beta (Tyr 431), murine adenine nucleotide translocase 1 (Tyr 81), bovine chymotrypsinogen (Tyr 201) and porcine pepsin (Tyr 310). Only 1–3 tyrosines per protein were modified, suggesting that the reactive tyrosine was activated by nearby residues that facilitated ionization of the hydroxyl group of tyrosine. These results suggest that OP binding to tyrosine is a general phenomenon. It is concluded that organophosphorus-reactive proteins include not only enzymes in the serine hydrolase family, but also proteins that have no active site serine. The recognition of a new OP-binding motif to tyrosine suggests new directions to search for mechanisms of long-term effects of OP exposure. Another application is in the search for biomarkers of organophosphorus agent exposure. Previous searches have been limited to serine hydrolases. Now proteins such as albumin and keratin can be considered.

1. Introduction

Organophosphorus (OP) agents are used as insecticides, fuel additives, plasticizers, lubricants, flame retardants, and chemical warfare agents [1,2]. These compounds are toxic to insects, fish, birds and mammals. Exposure can lead to a variety of symptoms culminating in seizures, respiratory arrest and death in acute cases [3]. The traditional targets for organophosphorus agents have long been considered to be the active site serine residues in acetylcholinesterase and butyrylcholinesterase. It is generally accepted that covalent inhibition of acetylcholinesterase is responsible for most of the clinically relevant symptoms observed upon high dose exposure to OP [3]. However, evidence has been accumulating over the past several years that suggests cholinesterases are not the only clinically relevant targets for OP, especially during low-dose exposure [1,2,4,5].

Investigations in several laboratories have been directed at identifying other proteins with which OP can react covalently. In addition to a variety of serine hydrolases [2,6], reactions of OP with other classes of enzymes and with receptors have been reported [2,7]. Results from our laboratory have demonstrated that non-enzymatic proteins such as transferrin [8], serum albumin [9–11] and tubulin [12] can be covalently modified by OP. For these latter proteins, the reactive amino acid is tyrosine.

Reaction of the organophosphorus agent diisopropylfluorophosphate (DFP) with a tyrosine residue in human serum albumin (and in bovine serum albumin) was reported by Sanger in 1963 [13]. Between 1965 and 1971, DFP was shown to react with tyrosine residues on bromelain [14], papain [15], and lysozyme [16].

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These findings were consistent with the known reactivity of tyrosine with organophosphorus agents [17]. Interest in the reaction of OP with protein-bound tyrosyl residues appears to have waned after 1970. However, starting in 1999, a resurgence of interest was re-kindled with the recognition that serum albumin provided an alternative to butyrylcholinesterase as a biological marker for exposure to OP [18]. Researchers responsible for this work took advantage of improvements in mass spectrometry that simplified the identification of post-translational modifications on proteins. Subsequent studies on serum albumin (1) identified Tyr411 as the most reactive tyrosine residue on human serum albumin, confirming Sanger's assignment [9]; (2) demonstrated that multiple tyrosine residues from albumin could react with OP [19]; and (3) showed that OP-labeled tyrosine could be detected in rodents that had been treated with sub-lethal doses of OP [11] including nerve agents [20].

To our knowledge, reaction of OP with tyrosyl residues has not been confirmed for any proteins other than those mentioned. If this reaction is wide spread, as we suspect, then it opens a new arena for investigation when considering intoxication due to OP exposure. Reactions with tyrosine could be responsible for intoxication that is not consistent with inhibition of acetylcholinesterase. Demonstrating that tyrosine residues from a wide variety of proteins will react with OP is an essential step for the development of this concept.

In this work, we have expanded the list of proteins that react with OP at tyrosine to include: human alpha-2-glycoprotein 1 zinc, human kinesin 3C, human keratin 1, bovine actin, murine ATP synthase beta, murine adenine nucleotide translocase 1, bovine chymotrypsinogen and porcine pepsin. We suggest that covalent reaction of OP with tyrosine is a general phenomenon that can be expected to occur for a large number of proteins.

Our findings may have application to diagnosis and treatment of OP exposure. Proteins that have no active site serine may serve as biomarkers of exposure. In the future it may be possible to develop antibodies to the new OP-labeled biomarkers to use for screening OP exposure. The recognition of a new OP-binding motif to tyrosine suggests new directions to search for mechanisms of long-term effects of OP exposure.

2. Materials and methods

2.1. Materials

Human alpha-2-glycoprotein 1 zinc was isolated from plasma. Human kinesin KIF3C motor domain was from Cytoskeleton Inc. (Denver, CO #KF01). Human epidermal keratin (#K0253), bovine actin (#A3653), bovine DNase (#D4527), porcine pepsin (#P6887), chicken lysozyme (#L6876), bovine RNase A (#R5125), bovine insulin (#I5500), diisopropylfluorophosphate (#D0879) and iodoacetamide (#I6125) were from Sigma (St. Louis, MO). Human IgG was from Fluka/Sigma (St. Louis, MO, #56834). ATP synthase beta and adenine nucleotide translocase 1 were isolated from a mouse heart membrane preparation. Porcine gelatin was from USB (Cleveland, OH, #16045). Chymotrypsinogen was found as a component of the bovine DNase preparation. Sequencing grade modified trypsin (#V5113, porcine, reductively methylated, TPCK treated) was from Promega (Madison, WI). Dithiothreitol was from Fisher Biotech (Fair Lawn, NJ, #BP172-25, electrophoresis grade). Alpha-cyano-4-hydroxy cinnamic acid (CHCA) from Fluka (#70990) was recrystallized before use then dissolved to 10 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid. NeutrAvidin agarose beads were from Thermo Scientific (Rockford, IL, #29202). Monomeric avidin agarose beads were from Pierce (Rockford, IL, #20228)

FP-biotin was custom synthesized in the laboratory of Dr. Charles M. Thompson at the University of Montana (Missoula, MT) [22]. Stock solutions of FP-biotin were made in dimethylsulfoxide and stored at −80 °C.

2.2. Sample preparation for mass spectrometry

Proteins were either purchased and were therefore relatively pure, or they were isolated from crude extracts. All pure proteins except kinesin were treated with FP-biotin by the following protocol. One mg/ml protein (approximately 10–25 μM) was dissolved in 10 mM ammonium bicarbonate, pH 8.3, containing 0.01% sodium azide. FP-biotin was added to a final concentration of 120 μM and the solution was incubated at 37 °C for 24 h. Negative controls were processed in the same manner as labeled samples except that FP-biotin was omitted. Samples were boiled for 10 min, reduced with 10 mM dithiothreitol for 2 h at 60 °C, alkylated with 50 mM iodoacetamide for 1 h at room temperature in the dark, and dialyzed against 4 l of 10 mM ammonium bicarbonate pH 8.3 for 24 h at 8 °C (with one change), and then digested with sequencing grade trypsin at a ratio of 1:100 (μg trypsin:μg sample) for 24 h at 37 °C. Digests were used directly for MALDI TOF TOF analysis or dried and resuspended in 5% acetonitrile, 0.1% formic acid for QTrap analysis.

Kinesin motor domain (1 mg or 13.3 nanomoles) was dissolved in 1 ml of 80 mM PIPES buffer pH 7.0 containing 0.5 mM EGTA, 2 mM $MgCl_2$, 0.2 M NaCl and 20 μM ATP. FP-biotin (260 nanomoles) was added. The protein did not fully dissolve. The mixture was incubated at 37 °C for 48 h, with occasional mixing. The protein was denatured in 8 molar urea in the presence of 10 mM dithiothreitol and boiled for 3 min. The resultant solution was clear. Sulfhydryl groups were alkylated with 90 mM iodoacetamide at 37 °C for 3 h. The protein solution was freed of unbound FP-biotin and salts by dialysis against 4 l of 25 mM ammonium bicarbonate, pH 8.5 for 36 h at 4 °C (with three changes). There were some particles in the preparation after dialysis. The preparation was then digested with trypsin (at a ratio of 50–1, μg protein to μg trypsin) for 11 h at 37 °C before a second aliquot of trypsin was added for another 14 h of digestion. Trypsin was inactivated by reaction with 1 μl of 5.73 M diisopropylfluorophosphate overnight. Labeled peptides were enriched by extraction with monomeric avidin beads (1 ml, settled volume) equilibrated in 10 mM ammonium bicarbonate, pH 8.5. Loaded beads were washed with 10 ml of 1 M potassium phosphate, pH 7.5; 10 ml of 0.1 M Tris acetate, pH 8.6; and 30 ml of water before elution with 10% acetic acid. The digest was examined with the MALDI TOF TOF 4800 mass spectrometer. Peptides that showed characteristic masses for FP-biotin under MSMS conditions were further purified by reverse phase HPLC (Phenomenex Prodigy 5 micron ODS column) using an acetonitrile/trifluoroacetic acid gradient. Fractions containing masses consistent with the putative FP-biotinylated peptides were dried, resuspended in 50% acetonitrile/0.1% formic acid, and infused into the QTrap 2000 mass spectrometer for improved MSMS fragmentation.

The FP-biotinylated tryptic peptide from human alpha-2-glycoprotein 1 zinc-binding protein was isolated from human plasma by extraction with NeutrAvidin Agarose beads as described [19].

Mouse heart membrane was prepared in 5 mM HEPES buffer, pH 7.4, from 5 mouse hearts as described [7]. The membrane preparation (at 2 mg/ml protein) was incubated with 100 μM FP-biotin at 37 °C overnight, then it was denatured with 8 M urea, reduced with 10 mM dithiothreitol for 1 h at 37 °C, alkylated with 40 mM iodoacetamide for 1 h at 37 °C in the dark, and dialyzed against 10 mM ammonium bicarbonate buffer. Tryptic digestion was accomplished by incubation at 37 °C overnight with Promega sequencing grade trypsin, at a trypsin to protein ratio of 1–37 (weight to weight). FP-biotinylated peptides were extracted from the digest with NeutrAvidin Agarose beads (1 ml settled volume) in 8 mM sodium phosphate buffer, pH 7.0, containing 155 mM sodium chloride (PBS).

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Beads were washed with 5–10 ml of PBS and then with 10 ml of water. Peptides were eluted with 50% acetonitrile/water containing 0.15% trifluoroacetic acid. It had been anticipated that this procedure would yield FP-biotinylated muscarinic receptor. However, the FP-biotinylated proteins identified in the preparation were ATP synthase beta and adenine nucleotide translocase I.

2.3. MALDI TOF TOF analysis

Generally, 0.5–1 μl of sample (approximately 20 pmole/μl for pure proteins, assuming no losses during processing) was air dried onto a 384-well Opti-TOF sample plate (Applied Biosystems, Foster City, CA, #1016491) and then overlaid with 1 μl of CHCA (10 mg/ml). Mass spectra and collision induced MSMS spectra were collected in positive ion reflector mode on a MALDI TOF TOF 4800 mass spectrometer (Applied Biosystems). The final spectrum was the average of 500 laser shots. The mass spectrometer was calibrated before each use with CalMix 5 (Applied Biosystems).

2.4. QTrap analysis

For analysis using on-line HPLC separation of sample before introduction into the mass spectrometer, a 1 ml sample from the tryptic digest was dried by SpeedVac and re-dissolved in 0.4 ml of 5% acetonitrile containing 0.1% formic acid to yield approximately 3–5 pmole of peptide/μl, assuming no losses during processing. Ten microliters of this solution (30–50 pmole, assuming no losses during handling) were injected onto an HPLC nanocolumn (218MS3.07515 Vydac C18 polymeric reverse phase, 75 μm I.D.—150 mm long; P.J. Cobert Assoc, St. Louis, MO). Peptides were separated with a 90 min linear gradient from 5 to 60% acetonitrile at a flow rate of 0.3 μl/min and electrosprayed through a fused silica emitter (360 μm O.D., 75 μm I.D., 15 micron taper, New Objective, Woburn, MA) directly into the QTRAP 2000, a hybrid quadrupole linear ion trap mass spectrometer (Applied Biosystems). An ionspray voltage of 1900 V was maintained between the emitter and the mass spectrometer. Information dependent acquisition was used to collect MS, high resolution MS, and MSMS spectra. All spectra were collected in the enhanced mode, using the trap function. The three most intense MS peaks in each cycle with mass between 400 and 1700 *m/z*, charge of +1 to +4, and intensities greater than 10,000 cps were selected for high resolution MS and MSMS analysis. Precursor ions were excluded for 30 s after one MSMS spectrum had been collected. The collision cell was pressurized to 40 μTorr with pure nitrogen, and collision energies between 20 and 40 eV were determined automatically by the software based on the mass and charge of the precursor ion. The mass spectrometer was calibrated on selected fragments from the MSMS spectrum of Glu-fibrinopeptide B.

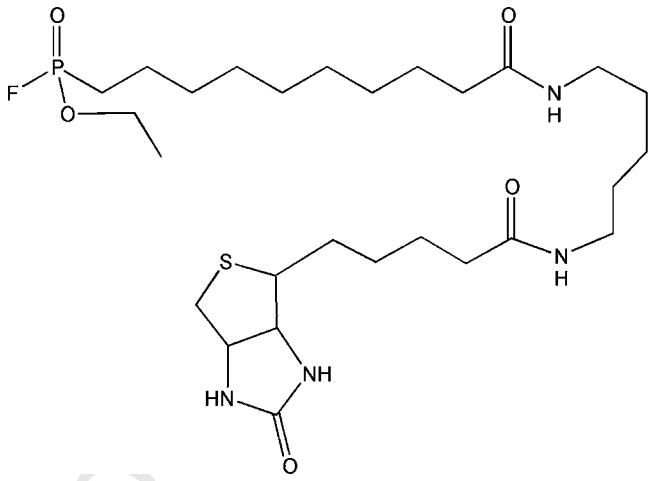

Fig. 1. The structure of FP-biotin, 10-(fluoroethoxyphosphinyl)-N-(biotinamidopentyl) decanamide.

The MSMS data were submitted to Mascot (Matrix Science, London, UK, http://www.matrixscience.com) for identification of labeled peptide [23]. The added mass of the FP-biotin (572 amu) has been incorporated into the UNIMOD database (http://www.unimod.org) for use as a variable modification in the Mascot algorithm. In addition, the MSMS chromatograms were searched with an extracted ion chromatographic protocol (Analyst 1.4.1 software, Applied Biosystems) for fragment ions at 329, 312 and 227 amu which are characteristic of the FP-biotin label [24]. Sequences of peptides identified by either procedure were confirmed manually.

3. Results

The structure of FP-biotin is shown in Fig. 1. FP-biotin was used for these studies for two reasons. First, MSMS fragmentation creates fragments that are characteristic of FP-biotin. Characteristic fragments are found at 329, 312 and 227 amu (for fragmentation of FP-biotin at its internal amide linkages) [24]. In addition, tyrosine-FP-biotin immonium ion fragments are found at 708 amu (for the singly charged immonium ion), 691 amu (for the immonium ion less amine), 355 amu (for the doubly charged immonium ion) and 346 amu (for the doubly charged immonium ion less amine). These ions provide a convenient, positive identification for the labeled

Table 1
FP-biotin labeled peptides.

Species	Protein	Accession number gi	Peptide sequence[a]	Tyr	FP-biotin characteristic ions				
					329	312	227	691	708
Human	Alpha-2-glycoprotein 1 zinc	52790422	WEAEPVY*VQR	174	X	X	X	X	X
	Alpha-2-glycoprotein 1 zinc	52790422	AY*LEEECPATLR	181	X	X	–	X	X
	alPha-2-glycoprotein 1 zinc	52790422	YY*YDGKDYIEFNK	138	X	X	X	X	X
	Kinesin 3C	41352705	ASY*LEIYQEEIR	145	X	X	X	X	X
	Keratin 1	119395750	THNLEPY*FESFINNLR	230	X	X	–	X	X
Bovine	Actin	62287933	DSY*VGDEAQSK	55	X	–	–	X	X
	Actin	62287933	GY*SFVTTAER	200	X	X	X	X	X
	Chymotrypsinogen	194674931	Y*TNANTPDR	201	X	X	X	X	X
Mouse	ATP Synthase beta	20455479	ILQDY*K	431	X	X	X	X	X
	Adenine Nucleotide Translocase 1	902008	Y*FPTQALNFAFK	81	X	X	X	X	X
Porcine	Pepsin	13096225	QYY*TVFDR	310	X	X	X	X	X

[a] The asterisk (*) indicates the labeled tyrosine. The residue number of the labeled tyrosine in the protein sequence is given. X indicates the presence of FP-biotin characteristic ions in MSMS spectra of the labeled peptide.

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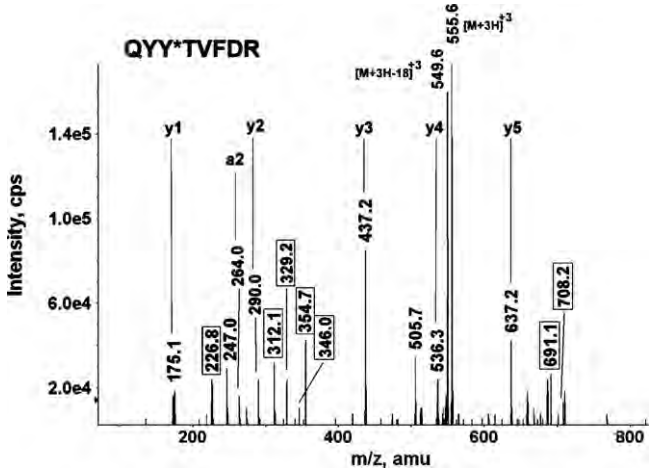

Fig. 2. The MSMS spectrum of peptide QYY*TVFDR from porcine pepsin (where Y* is the labeled tyrosine). The boxed masses identify the non-sequence, characteristic fragments from FP-biotin. The triply charged parent ion has a mass of 555.6 amu.

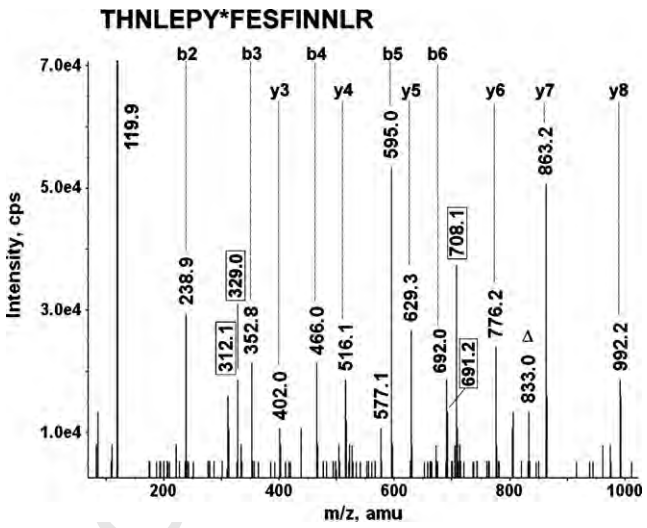

Fig. 4. The MSMS spectrum of peptide THNLEPY*FESFINNLR from human keratin 1 (where Y* is the labeled tyrosine). The boxed masses identify the non-sequence, characteristic fragments from FP-biotin. The mass marked with a triangle (833.0 amu) is the N-terminus of a proline internal fragment. The triply charged parent ion has a mass of 642.6 amu which does not appear in the spectrum.

peptides. They also can be used as target masses when the mass spectra from crude mixtures are searched for the labeled peptides using extracted ion techniques. Second, avidin agarose can be used to concentrate and enrich the biotinylated-peptides from crude mixtures.

In the current study, eleven peptides from eight proteins were found to be labeled (see Table 1). The parent ion mass of each peptide was consistent with a covalent reaction of FP-biotin with some residue on that peptide (added mass of 572 amu). As it turned out, each peptide was labeled on tyrosine. The labeled residue for each peptide was confirmed by manual analysis of the MSMS spectra, with the assistance of the MS-Product algorithm for assignment of internal fragments (University of California, San Francisco, http://prospector2.ucsf.edu). Four representative MSMS spectra are presented.

Fig. 2 shows the MSMS spectrum of QYY*TVFDR from porcine pepsin where FP-biotin is on Tyr 310. A 5-residue y-ion sequence could be extracted which included the C-terminal arginine. The labeled tyrosine was part of an unresolved three amino acid y-ion fragment at the N-terminus. This three amino acid fragment contained two tyrosines. Fortunately, masses consistent with the unlabeled a2-ion (QY = 264.0 amu) and its deaminated consort at 247.0 amu appeared in the spectrum. Thus, by a process of elimination the label could be assigned to the tyrosine in position three from the N-terminus. The majority of the remaining masses in the spectrum could be assigned to dehydration/deamination products or internal fragments. Characteristic fragments from FP-biotin appeared at 226.8, 312.1, 329.2, 708.2, 691.1, 354.7 and 346.0 amu.

Fig. 3 shows the MSMS spectrum of GY*SFVTTAER from bovine actin where FP-biotin is on Tyr 200. The peptide was identified by an eight amino acid y-ion sequence. The labeled tyrosine appeared in all b-ions, for example in the singly charged b2-ion at 793.2 amu and in the doubly charged b2-ion at 397.3 amu. The majority of the remaining masses in the spectrum could be assigned to dehydration/deamination products or internal fragments. Characteristic fragments from the internal amide bonds of FP-biotin appeared at 226.8, 312.2, 329.4, 708.4, 691.2, 355.0 and 346.3 amu.

Fig. 4 shows the MSMS spectrum of THNLEPY*FESFINNLR from human keratin 1 where FP-biotin is on Tyr 230. The peptide was identified by an eight amino acid y-ion sequence and a six amino acid b-ion sequence. The label was found as part of the Pro Tyr pair (833.0 amu) at the N-terminus of the proline internal fragment. The majority of the remaining masses in the spectrum could be assigned to dehydration/deamination products or internal

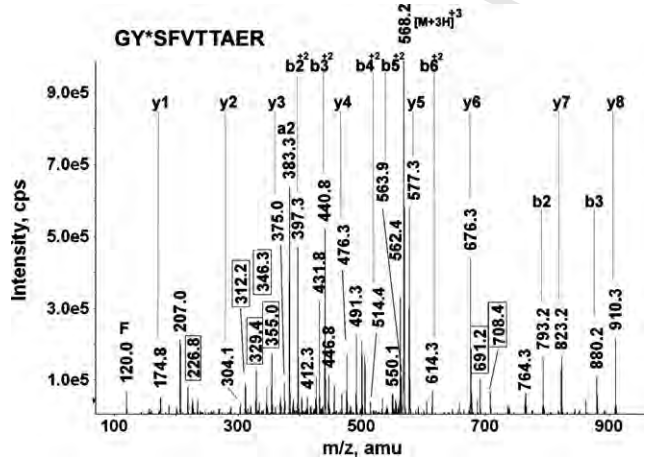

Fig. 3. The MSMS spectrum of peptide GY*SFVTTAER from bovine actin (where Y* is the labeled tyrosine). The boxed masses identify the non-sequence, characteristic fragments from FP-biotin. The triply charged parent ion has a mass of 568.2 amu.

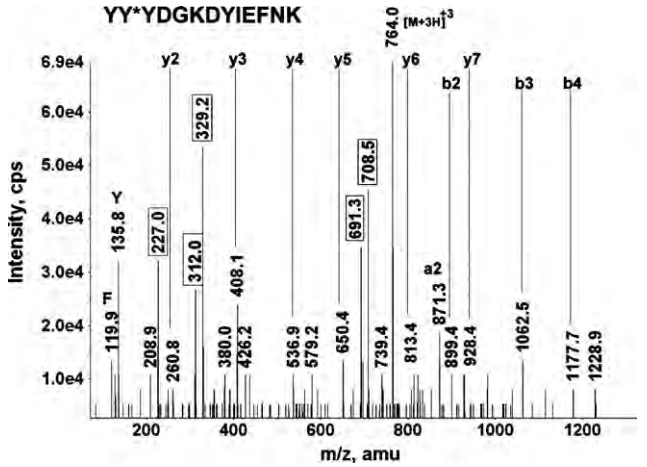

Fig. 5. The MSMS spectrum of peptide YY*YDGKDYIEFNK from alpha-2-glycoprotein 1 zinc-binding protein (where Y* is the labeled tyrosine). The boxed masses identify the non-sequence, characteristic fragments from FP-biotin. The triply charged parent ion has a mass of 764.0 amu.

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fragments. Characteristic fragments from the internal amide bonds of FP-biotin appeared at 312.1, 329.0, 708.1 and 691.2 amu.

Fig. 5 shows the MSMS spectrum of YY*YDGKDYIEFNK from human alpha-2-glycoprotein 1 zinc-binding protein where FP-biotin is on Tyr 138. Though the signal-to-noise ratio was low, the peptide could still be identified by a seven amino acid y-ion sequence and a four amino acid b-ion sequence. The minimum signal-to-noise ratio for the sequence peaks had an acceptable value of three. The label was found as part of a trimer at the N-terminus that consisted of three tyrosines. Analysis of the b-ion sequence demonstrated that the label was on the b2-ion (899.4 amu) and therefore had to be on one of the first two N-terminal tyrosines. Though the b2 peak had low intensity, its identity was confirmed by the a2-ion at 871.4 amu which was substantially more intense. Since it could not be determined which tyrosine actually carried the label, the second tyrosine was arbitrarily marked as labeled. Characteristic fragments from the internal amide bonds of FP-biotin appeared at 227.0, 312.0, 329.2, 708.5 and 691.3 amu.

Labeled amino acids in the seven remaining peptides were identified using similar analyses of their MSMS spectra. The label was found as one of the sequence fragments in peptide WEAEPVY*VQR. The labeled amino acid was found as part of the b2-ion in peptides AY*LEEECPATLR and Y*FPTQALNFAFK. For both sequences, the only reasonable candidate for labeling was the tyrosine. For peptide ILQDY*K, the labeled tyrosine was part of the y2-ion at the C-terminus. Again, the only reasonable candidate for labeling was the tyrosine. If the lysine had been labeled, it would not have been recognized for cleavage by trypsin. The labeled tyrosine was part of a y-ion dimer at the N-terminus of peptide Y*TNANTPDR and as part of an unresolved three amino acid y-ion fragment at the N-terminus of two sequences (ASY*LEIYQEEIR and DSY*VGDEAQSK). In principle, either serine or threonine might have been labeled rather than tyrosine in all three peptides. However, there are three lines of evidence to indicate that the label is on tyrosine rather than serine or threonine. First, there was no evidence for the 591 amu fragment that is characteristic of a serine label (and presumably of a threonine label) [24]. Second, OP-labeled serine (like phosphoserine) readily loses the OP-label in the mass spectrometer, under the CID conditions employed in these experiments. This fragmentation leaves a dehydroalanine residue in its stead. A similar loss for OP-threonine would be predicted to yield dehydro-threonine. The masses of the observed fragments were consistent with the presence of both the intact serine (or threonine) and the OP label. No masses consistent with the loss of OP and the presence of either dehydroalanine or dehydro-threonine were detected. Third, masses for both tyrosine-FP-biotin immonium ions and tyrosine-FP-biotin immonium ions minus amine were detected in all three MSMS spectra, indicating the presence of FP-biotinylated tyrosine. For these reasons, the label was assigned to tyrosine in each case.

Non-sequence fragments characteristic of FP-biotinylated tyrosine at 329, 312, 691, 708 and 355 amu masses appeared in all 7 MSMS spectra and the 346 amu mass in 5 spectra. The presence of these ions in the MSMS spectra confirmed the presence of FP-biotin on the peptides.

Not all of the proteins that we treated with FP-biotin yielded labeled peptides. Proteins for which we could find no FP-biotinylated peptides included porcine gelatin, bovine RNase, bovine DNase I, human IgG, chicken lysozyme, and bovine insulin.

4. Discussion

4.1. General phenomenon

We have previously demonstrated that tyrosine in transferrin, serum albumin and tubulin (both alpha and beta) react in vitro with a variety of organophosphorus agents (FP-biotin, chlorpyrifos-oxon, diisopropylfluorophosphate, dichlorvos, soman, and sarin) [8–10,12,19]. In addition, SDS PAGE experiments have demonstrated the presence of at least 50 FP-biotin reactive proteins in mouse brain supernatant, again in vitro [25]. Many of these latter proteins were identified as members of the serine hydrolase family. Such proteins are the expected targets for FP-biotin [26]. However, many were not members of that family [25].

The current experiments clearly demonstrate that the organophosphorus agent FP-biotin is capable of reacting with tyrosines on a variety of non-serine hydrolase proteins. By inference, other OP should react with these tyrosines as well.

With the addition of these proteins to those that have already been identified, it stands to reason that the reaction of protein-bound tyrosine with FP-biotin is a widespread phenomenon. On the other hand, not all tyrosines in a protein are reactive nor do all proteins contain reactive tyrosines.

4.2. Only certain tyrosines react with OP

The question of what determines the selectivity for the reaction of some tyrosines with FP-biotin arises. In order for a tyrosine to react with FP-biotin, it must satisfy two criteria. First, the phenolic oxygen of the tyrosine must be exposed to the medium. The necessity for this criterion is self-evident.

Second, the phenolic oxygen must be capable of nucleophilic attack. Reactions of OP typically involve attack by a nucleophile on the phosphorous to displace the most labile of the OP ligands [27]. For tyrosine to serve as a nucleophile, it must be deprotonated at the pH of the reaction. Ashbolt and Rydon [17] have demonstrated that tyrosine alone can react with diisopropylfluorophosphate (13 mM diisopropylfluorophosphate, 3 mM tyrosine, at pH 7.8 and 37 °C for 24 h). Despite the fact that the concentration of OP in their experiments was 100-fold higher than in ours they demonstrated the potential of tyrosine to react with OP.

The high concentrations of OP used by Ashbolt and Rydon were necessary because of the low reactivity of tyrosine under their conditions. This low reactivity can be attributed to the pK_a of tyrosine. The pK_a of tyrosine is 10.1 [28]. At pH 8.3, where our reactions were conducted, only 1% of the typical tyrosine would be deprotonated. To promote a nucleophilic reaction of protein-bound tyrosine with OP, some mechanism for increasing the amount of deprotonated tyrosine would be advantageous. A simple way to accomplish this objective would be to lower the pK_a value of the tyrosine.

It has been demonstrated that interactions between histidine and cysteine provide a viable method for lowering the pK_a of cysteine in proteins. Decreases of 4–5 pH units have been reported [29,30]. It has been proposed that the decrease in cysteine pK_a is due to through-space, charge–charge interactions [31]. Lowered pK_a values for tyrosine in human transferrin [32] and UDP-galactose 4-epimerase [33] also have been reported. The decrease in pK_a was ascribed to interaction with nearby positively charged residues. In the case of UDP-galactose 4-epimerase, NAD^+ was implicated. For human transferrin, four tyrosines were found to have pK_a values around 7. Neighboring lysines were implicated. We suggest that this sort of process may be involved in activating the FP-biotin reactive tyrosines in the peptides described in this paper.

4.3. Labeled peptides are hard to find; knowing what to look for helps

Our ultimate goal is to identify the proteins that are modified by low-dose exposure in an animal, and eventually in humans. Organophosphorus pesticides have no tag to help identify the labeled peptide. The task is to find a labeled peptide based on

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its mass, starting from a mixture of 30,000 proteins which upon digestion with trypsin will yield at least a million peptides. This is a very difficult assignment. The work we are doing with pure proteins aims to make it possible. By knowing what we are looking for, we can reduce the complexity of the starting material. For example, the most OP-reactive protein in human plasma is butyrylcholinesterase. By purifying butyrylcholinesterase from plasma before beginning a search for the labeled peptide, one can successfully find the labeled peptide and identify the modifying agent [34,35].

Covalent modification of butyrylcholinesterase and acetylcholinesterase does not explain cognitive impairment and depression following pesticide exposure [36–38]. Therefore we are searching for unknown proteins modified by OP. When we first identified FP-biotinylated albumin in mice treated with a nontoxic dose of FP-biotin [11], and identified Tyr 411 of human albumin as the site modified by OP [9], we thought the OP-tyrosine adduct on albumin was an exception. Only with study of additional proteins has it become clear that covalent binding of OP to tyrosine is common. This principle allows us to expand our search. One way we have applied this principle is by adding OP-bound tyrosine as a variable modification in the UNIMOD database (http://www.unimod.org) of the Mascot search engine (http://www.matrix-science.com); all Mascot users can now search for OP-tyrosine adducts.

Knowing the exact mass of candidate OP-labeled peptides helps us find the labeled peptide. Even after a protein has been partially purified from plasma or from brain, there are still thousands of peptides in the tryptic digest. In a mixture of peptides some peptides dominate in a process called ion suppression, making it impossible for other ions to ionize in the mass spectrometer. Often, the labeled peptide cannot be found. An example of the ion suppression problem and the difficulty of finding a labeled peptide is the fact that we could not find the labeled peptide in pure chicken lysozyme even though it is known that tyrosine in chicken lysozyme is labeled by OP [21]. The solution to the problem of ion suppression is to fractionate the peptides. We separate the peptides offline by HPLC and check each fraction by MALDI-TOF to identify the fraction that includes the mass of interest. This step requires that we know the mass we are looking for. This is where our studies with pure proteins are very helpful. The partially purified peptide is then subjected to LC/MS/MS which adds another liquid chromatography purification step before the peptide is fragmented in the mass spectrometer. We search the data for labeled peptides using Mascot software, but we also search the data manually for peptides that Mascot might have missed. Mascot generally does not report peptides that contain fewer than 5 amino acids in its search results. For example, the OP-labeled tryptic peptide of bovine albumin YTR, was not reported by Mascot but it was found by manual examination of the data [24]. This step requires that we know what we are looking for. In conclusion, the information obtained from a study of pure proteins labeled with OP is the basis for in vivo studies that aim to identify proteins modified by OP.

4.4. In vivo studies

The issue that we currently have under investigation is whether reaction of OP with proteins occurs in vivo at OP concentrations low enough that signs of cholinergic toxicity do not appear. We have already demonstrated that albumin and carboxylesterase from mouse plasma together with nine other unidentified proteins can be labeled in vivo by FP-biotin, at concentrations that do not significantly inhibit acetylcholinesterase [11]. The reactive residue on albumin is tyrosine [9,20], while that on carboxylesterase is the active site serine [39]. In vivo studies in guinea pigs treated with soman and tabun identified OP-tyrosine adducts on albumin [20]. In vivo studies to search for other proteins modified by OP are underway. The work with pure proteins in this report will aid in the identification of proteins modified in vivo.

4.5. Significance

Our findings may have application to diagnosis of OP exposure. Proteins that have no active site serine may serve as biomarkers of exposure. In the future it may be possible to develop antibodies to new OP-labeled biomarkers to use for screening OP exposure. The recognition of a new OP-binding motif to tyrosine suggests new directions to search for mechanisms to explain cognitive deficits and depression associated with exposure to OP.

Conflict of interest

The authors declare that there are no conflicts of interest.

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